THE AMERICAN JOURNAL OF PHYSIOLOGY

EDITED FOR

THE AMERICAN PHYSIOLOGICAL SOCIETY

CONTENTS

VOL. 113—NO. 3

Issued November 1, 1935

BALTIMORE, U. S. A.

1935

Entered as second-class matter, August 18, 1914, at the Post Office in Baltimore, Md., under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917. Authorized on July 5, 1918

Made in United States of America

THE AMERICAN JOURNAL OF PHYSIOLOGY

Editorial Policy. The Council has approved the following policy of management:

Manuscripts should be sent to the Managing Editor who will see that each paper is read by two or more members of the Editorial Board. Authors will then be advised as to the suitability of the paper or the desirability of any revision. The Editorial Board will be governed by certain general principles:

1. The suitability of papers will not be judged by arbitrary standards of length but on their content of significant new research results in physiology, presented with the greatest brevity which is compatible with scientific accuracy and clarity.

2. Preference will be given to papers from American Laboratories in the field of vertebrate physiology and to those which contribute to problems related to this field.

3. Subdivision of material coming out of a general research into two or more papers will be discouraged.

4. Papers restricted to the description of new apparatus or methods or which appear to be of the nature of progress reports, the publication of which might properly be withheld until the research has progressed to the completion of at least a significant phase of the problem, will not be accepted.

5. Papers giving confirmatory or negative results will be considered only if presented in the briefest possible space.

6. Since manuscripts will not be insured against loss or injury when being given editorial consideration, contributors will be expected to retain duplicate copies of all material (manuscripts, illustrative and tabular matter) submitted for publication.

The following practical aspects are important in the preparation of papers:

a. Duplication of data in tables, charts and protocols is not believed to be generally necessary. Too extensive use of such material is likewise to be deprecated.

b. Tables and illustrative material should be prepared with the size of the Journal page (4½ x 7½ inches) in mind, specifically with the idea of conserving vertical space.

c. It is advantageous, when feasible, to group illustrations. This should be done with as little waste space between the several units as is possible and also with the idea of conserving vertical space.

d. Since duplication of charts, graphic tracings, etc., is required in paragraph six above, this is best done by photographs of the size desired in reproduction and printed on glossy paper. When these are adequate for good reproduction the originals need not be supplied.

e. Plotted curves and their guide-lines should be drawn in India ink on blue-lined coördinate paper.

f. All illustrative material must be submitted in such form as to admit of photographic reproduction without retouching, redrawing or the setting of marginal type.

g. References to cited papers should conform to the practice of the Quarterly Cumulative Index Medicus and should be in this order: Author's name, journal, volume (in Arabic), initial page, year.

The American Journal of Physiology is issued monthly by the American Physiological Society under the direction of the Council of the Society. From three to four volumes, each of about seven hundred and twenty pages, are published yearly. The subscription price per volume in the United States and Canada is $7.50; in other countries, $8.00.

THE AMERICAN
JOURNAL OF PHYSIOLOGY

VOL. 113 NOVEMBER 1, 1935 No. 3

FACILITATION OF MOTONEURONES[1]

R. LORENTE DE NÓ

From the Central Institute for the Deaf, St. Louis, Missouri

Received for publication June 3, 1935

The phenomena of facilitation of motoneurones, as they appear in the spinal reflex arc, have been very thoroughly studied (modern reviews in Fulton, 1926; Sherrington, 1925, 1929; Creed, Denny Brown, Eccles, Liddell and Sherrington, 1932; Forbes, 1934). The results reported in this paper largely agree with the observations of previous authors, but in some points are very different, undoubtedly because the preparation used is much simpler than the ordinary reflex arc and the impulses do not need to pass through primary sensory nuclei.

TECHNIQUE. The preparation used was described in a previous paper (Lorente de Nó, 1935a). Bipolar stimulating electrodes were placed on the floor of the fourth ventricle, in order to set up impulses in paths which end on the motoneurones of the third nucleus; the action potentials developed by the responses of the left internal rectus muscle were recorded with a cathode ray oscillograph.

The stimulating shocks were produced by discharge of condensors of about $0.1\mu f$ through a Harvard coil with the secondary coil over the primary. The duration of their negative wave was no more than 0.07σ so that it was possible to set two full sized shocks through the same coil at any interval of more than 0.1σ.

Since both shocks (the first or conditioning and the second or testing) had to be produced by the same stimulator their relative strength was regulated by changing the capacity of the first condensor from 0.04 to $0.14\mu f$, the capacity of the second condensor being maintained constant at $0.1\mu f$. Measurements with the cathode ray oscillograph have shown that the voltage of the shock is roughly proportional to the capacity of the condensor.

[1] The work reported in this paper has been aided by a grant from the Rockefeller Foundation.

RESULTS. *The nature of the response of the motoneurones to impulses set up in secondary paths by a single shock delivered through electrodes (F) placed on the floor of the fourth ventricle.* It was mentioned in a previous paper (Lorente de Nó, 1935a) that in a given preparation the strength of the response of the motoneurones to a shock through the *F* electrodes greatly depends on the state of the centres. This fact was interpreted as the proof of the existence during nystagmus of a subliminal fringe such as that found by Denny Brown and Sherrington (1928) in spinal reflexes. The great variability of the strength of the response in different preparations and in the same preparation after lesions in the brainstem suggests now the existence of a constant "tonic" subliminal fringe, so that the response of the motoneurones to single shocks is always due to summation of the preëxisting subliminal excitation and the excitation created by the shock.

It is a well known fact that the eye muscles are submitted to a constant labyrinthine innervation, which depends on the position of the head in space and may be roughly measured in terms of the isotonic shortenings of the eye muscles caused by changes of position of the head in space (Dusser de Barenne and de Kleyn, 1931; Lorente de Nó, 1925, 1932). Also the first three cervical nerves are a source of tonic impulses. The tonic innervation reveals itself as irregular potential waves (fig. 3, *10*) presumably due to asynchronous discharge of a few motor units, which can be led off from the muscles during absolute rest of the eye (Köllner and Hoffman, 1922, Bishop and Lorente de Nó, unpublished observations; Lorente de Nó, 1935d).

The tonic innervation is of course dependent on the impulses originated in the sensory endings of the labyrinth and of the neck muscles, but it seems that the impulses reaching the motor nuclei are chiefly produced by the chains of internuncial neurones of the reticular substance. The tonic innervation of the eye muscles persists after extirpation of both labyrinths (Köllner and Hoffmann, 1922) and although reduced also after destruction of the primary vestibular nuclei on both sides and after a transversal section of the dorsal third of the spinal cord between the first spinal segment and the medulla. However, the destruction of the reticular substance in medulla and pons abolishes it.

After that lesion, single shocks of any strength through the *F* electrodes remain ineffective, although two shocks in succession may produce very strong responses, even maximal twitches.

Discussion. The shock through the *F* electrodes stimulates fibres (*a* in fig. 1. Lorente de Nó, 1935a; *f* in fig. 4, I) which have synapses with the motoneurones of the third nucleus. The destruction of the reticular substance underneath and behind the electrodes does not prevent those impulses from reaching the motoneurones. Therefore the absence of the

response can be interpreted only as due to the disappearance of the facilitating effect of the tonic innervation by reticular neurones.[2]

This result throws further light on an important question. It was found (Lorente de Nó, 1928, 1931, 1933a; Spiegel, 1929) that the labyrinthine reflexes of the eye muscles may be obtained after complete section of all the long uninterrupted paths which connect the vestibular nuclei with the oculomotor ones, and it was concluded (Lorente de Nó, 1927, 1933a) that the impulses conducted by those paths remain subliminal for the motoneurones and produce only a facilitation; the actual response being due to the impulses arriving through internuncial paths. This assumption is now fully substantiated by experiment, although of course, the wording of it has to be changed, because any one set of impulses (the direct or the internuncial ones) may play the rôle of a facilitating excitation.

The paths stimulated by the *F* shock contain indeed many fibres, which establish a considerable number of synapses on the motoneurones. That in spite of it the stimulation of those paths may remain ineffective is an important fact. It seems as if effective summation of simultaneous impulses should demand special conditions; for instance, immediate proximity of the activated synapses or perhaps the stimulation of several kinds of synapses. That each neurone possesses several sets of synapses, often arranged in a very systematic manner is a fact which was object of a careful analysis (Lorente de Nó, 1933b, 1934b), and certainly must have physiological significance.

The response of the motoneurones to two shocks in quick succession through the F electrodes. In order to reach the floor of the fourth ventricle and place on it the stimulating electrodes the vermis of the cerebellum was extirpated. This, of course, changed the tonic innervation of the internal rectus muscle in a degree dependent on the amount of vestibular paths of the vestibulo-cerebellar complex damaged in the operation. When the operation was symmetrical the excitability of the preparation remained for a considerable length of time, generally during the whole experiment, at constant level, so that the height of the response to single shocks varied only by a few per cent; as a matter of fact it often was as constant as in the ordinary nerve muscle preparation.

However when the extirpation of the vermis was not symmetrical the preparation showed cyclic changes of excitability or even nystagmus. Generally, during the first two hours the spontaneous changes of excitability first decreased and then disappeared. During the initial state such

[2] A state of inhibition of the motoneurones could scarcely be assumed. The irresponsiveness appeared after the connections of the peripheral apparati and of a great part of the internuncial relays were destroyed and it remained without appreciable change for at least six hours.

preparations were not very suitable for the study of facilitation; but the destruction of the primary vestibular nuclei or if necessary also of the reticular substance in the medulla immediately suppressed any cyclic change of excitability and rendered a quiet preparation.

As a rule, after a first (conditioning) shock through the *F* electrodes the response to a second (testing) shock was modified during a certain period of time. Three different types of modification were found. 1. The conditioning shock was followed by a period of facilitation; 2, the conditioning shock was followed by a short period of facilitation and a longer one dur-

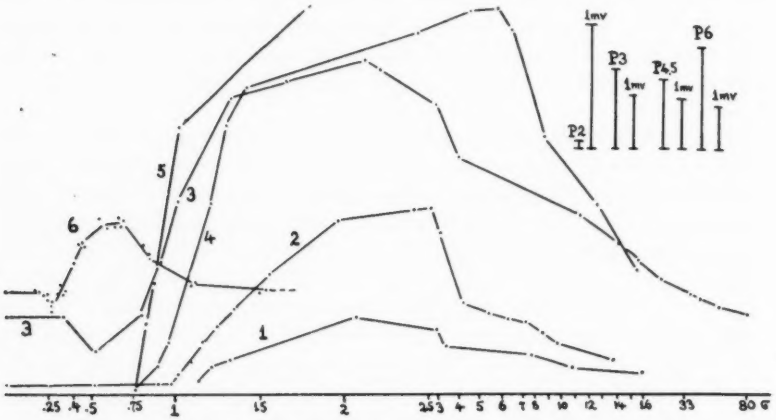


Fig. 1. Potential of the response to the testing *F* shock (ordinates) plotted against the interval in sigmata by which it follows the conditioning *F* shock (abscissae).

1–5. Experiment 11/18/34. Vestibular nuclei destroyed and besides a transversal section in pons as in figure 15 in Lorente de Nó, 1933a. *P 2*, *P 3* and *P 4*, 5 potential of the response to the unconditioned testing shock corresponding to the curves with the same numeral. The relations between the strength of conditiong and testing shocks were: 1, 40/100; 2, 50/100; 3, 40/100; 4, 160/100; 5, 120/100. In *4* the testing shock was just maximal, in *5*, supramaximal (125/100).

6. Experiment 11/19/34. Intact medulla. Conditioning shock just above threshold. Testing shock 250/100 larger.

ing which the testing shock elicited a series of responses; 3, the conditioning shock was followed by a first period of facilitation and a second one of inhibition. In this paper only the first two types will be considered.[3]

1. *The conditioning shock was followed by a period of facilitation of the response to the testing shock.* The phenomena of facilitation are best described by means of curves obtained by plotting height of the response to the testing shock against interval between shocks. For the sake of brevity

[3] The third type is described in another paper: "Inhibition of motoneurones" to be published in the symposium in honour of Professor Beritoff.

such curves will be referred to as "facilitation curves." Since the responses as a rule were practically synchronous twitches the recorded potentials were a fair indicator of the number of active motor units.

Several facilitation curves have been reproduced in figures 1 and 2. Figure 3 contains some of the records used in their construction. Curves *1, 2, 3* (fig. 2) illustrate facilitation when both shocks were subliminal,[4] the others when either one of the shocks or both were liminal. In obtaining curves *1, 2, 3* and *6* (fig. 1) and *1, 2* and *4* (fig. 2) the conditioning shock was made considerably smaller than the testing one; but in obtaining the other curves the testing shock was the smallest.

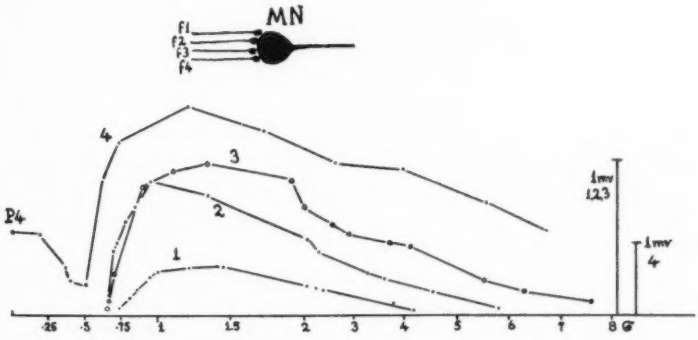


Fig. 2. Potential of the response to the testing *F* shock (ordinates) plotted against the interval in sigmata by which it follows the conditioning one (abscissae).

1, 2, 3. Experiment 3/12/34. Vestibular nuclei and reticular substance in medulla and pons destroyed. Both shocks subliminal when alone. Relation between conditioning and testing shocks: 1, 50/100; 2, 60/100; 3, 160/100.

4. Experiment 7/1/35. Intact medulla. Subliminal conditioning shock 60/100 of the testing one. *P 4* potential of the response to the unconditioned testing shock.

The relation between the strength of the smaller conditioning shock to the larger testing shock was chosen between 40 and 60/100 because it was determined with the nerve muscle preparation that such a testing shock readily overcame any post cathodal depression of fibres stimulated below threshold by the conditioning one. On the other hand when the conditioning shock was larger than the testing one there was no doubt that the latter could not stimulate any fibre which had not responded to the former.

In spite of considerable difference in details all the facilitation curves have the same fundamental traits.

[4] The denomination "subliminal stimulus" refers of course to the response of the motoneurones. Such a stimulus must have been liminal for some fibres and some internuncial neurones, otherwise it could not produce facilitation.

All the curves agree in that facilitation does not begin immediately after the conditioning shock, but after a certain interval of time, which may vary from 0.4σ to 1.10σ. This interval has proved to be quite independent from the absolutely refractory period of stimulated fibres.

When the conditioning shock was the largest one (curves *4*, *5*, fig. 1 and *3*, fig. 2) evidently the testing shock had to remain ineffective until at least a certain number of fibres had recovered from absolute refractoriness and were able to conduct a second impulse. In several experiments in which rather weak shocks were used a response to the testing shock was obtained at an interval between shocks of 0.52 and 0.62σ, so that it had to be concluded that at that interval a considerable number of fibres had recovered from absolute refractoriness. But in the experiment illustrated by records *1* to *6* in figure 3 and curves *4* and *5* in figure 1 the earliest response to the strong testing shock appeared after a much longer interval (0.75σ).

The experiments in which the conditioning shock was considerably smaller than the testing one were still more convincing. In some cases the earliest facilitated response was seen at an interval of 0.4σ to 0.43σ (fig. 1, curve *6*) i.e., before the stimulated fibres could have recovered from the absolute refractoriness; in other cases facilitation could not be demonstrated as late as 1.10σ after the conditioning shock (fig. 1, curve *1*), i.e., long after the stimulated fibres had recovered from refractoriness. Besides the beginning of facilitation was made to appear earlier by strengthening the conditioning shock (fig. 1, curves *1*, *2*, *3*; fig. 2, curves *1*, *2*), or by strengthening the testing one.

Another constant feature of the facilitation curves is their slow initial ascent. The extreme cases are represented by curves *6* and *1* in figure 1.

Fig. 3. *1–6* Experiment 11/18/34. Records used in the construction of curve 2, figure 1; *1*, testing shock alone ; *2–6*, both shocks at progressively increasing intervals. Facilitation begins in *4*; *7*, timing film (2000 cycles). *8* and *9*, potentials recorded from the oculomotor nucleus in the same experiment; in *8* subliminal shocks (the first one 250/100 larger) to show the shock artefacts; in *9*, the same shocks as in records *1–6*. *a*, shock artefact; *w 1*, *w 2*, potential waves; *10* and *11*, experiment 12/3/34, intact medulla. High amplification, which reveals the "tonic" waves in the resting muscle. In *10* a weak twitch (*t*) due to a small *F* shock does not modify appreciably the waves, but in *11* after a larger *F* shock the tonic waves are considerably increased during about 10σ, which closely corresponds to the period of facilitation in that experiment. *12–14*. Experiment 11/19/34, intact medulla. Records used in curve *6*, figure 1; *12*, testing shock alone; *13–14*, both shocks; facilitation begins in *14*; *15–18*, experiment 7/1/35, records used in curve *4*, figure 1; *15*, testing shock alone; *16–19*, both shocks, in *16* there is depression but in *17* and *19* the response to the testing shock is greatly facilitated; *18*, timing film (1000 cycles); *20–22*, experiment 11/1/35, intact medulla. *20*, testing shock alone, the response consists of two twitches; *21*, both shocks, the response consists of a practically synchronous twitch; *22*, timing film (1000 cycles).

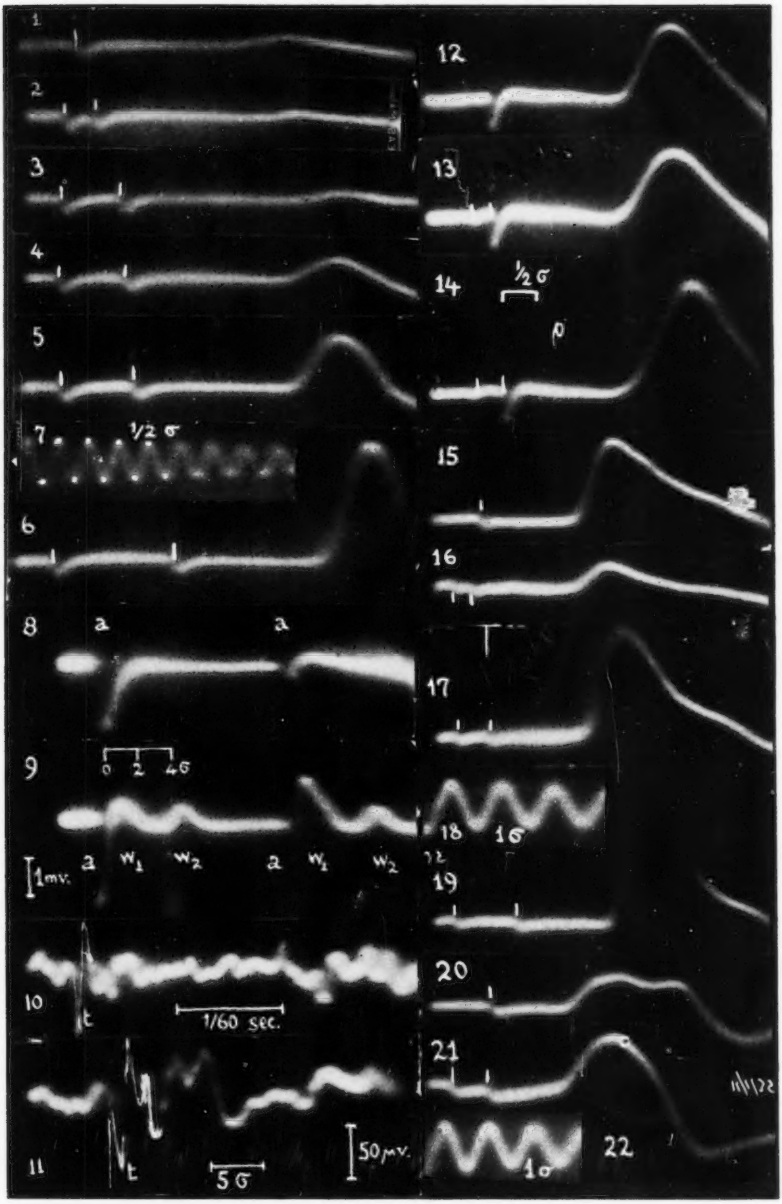

1
2
3
4
5
7
½ σ
6
8
a
a
9
0 2 4σ
1mv.
a
W1
W2
a
W1
W2
10
t
1/60 sec.
11
t
5σ
50μv.
12
13
14
½σ
p
15
16
17
18
1σ
19
20
21
22
1σ

Fig. 3

In some cases the slope of the ascending part of the curve may illustrate to a certain extent the statistical distribution of absolutely refractory periods of fibres stimulated by the conditioning shock. For instance the slope of curves *3* and *5* (fig. 1) obtained with a supramaximal testing shock is steeper than that of curve *4* obtained with a just maximal shock. But in the case of curve *6*, figure 1, where the ascent is very fast or in the cases of curve *1* and *2* where the ascent is very slow, it is evident that refractoriness of nerve fibres cannot have been the determining factor.

The maximum of facilitation was found sometimes as early as 0.70σ (curve *6*, fig. 1) and sometimes as late as 6σ (curve *4*, fig. 2) after the conditioning shock. As a rule in preparations with intact medulla it was found at 1.25σ to 1.5σ interval between shocks.

The duration of the period of facilitation was also variable within very wide limits (from 1.5σ to 80σ); in general it was lengthened by increasing the strength of the conditioning shock (curves *1* to *5* in fig. 1 and *1* to *3*, fig. 2). In preparations with intact medulla facilitation was demonstrable, as a rule, during from 7σ to 20σ.

Discussion. The mentioned features of facilitation (variable beginning of the period of facilitation independent of the absolute refractoriness of the stimulated fibres, slow ascent of the facilitation curve, late and variable maximum, long and variable duration) make it very improbable that facilitation could be due chiefly to summation, within the motoneurones themselves or in their immediate neighborhood, of quantums of an enduring excitatory process or of an excitatory chemical mediator, produced by the impulses set up in the stimulated fibres (*f* in fig. 4, *I*); they suggest that the facilitatory mechanism consists of internuncial relays, where repetitive volleys of facilitatory impulses are created.

The very important rôle of the internuncial relays of the vestibulo-ocular reflex arc was analysed in former papers (Lorente de Nó, 1928, 1931, 1933a) and recently Gasser and Graham (1933) and Hughes and Gasser (1934a) have furnished proof of the participation of the internuncial cells in the establishment of spinal reflexes. Among other things these authors found that weak shocks, which did not elicit any motor response, gave rise to internuncial potentials. Hughes and Gasser (1934b) have correlated the phenomena of facilitation and certain internuncial potentials.

A theory of the activity of the internuncial relays was developed in other papers (Lorente de Nó, 1932b, 1933a, 1934a, b). It was established in anatomical studies that the internuncial neurones are arranged in closed chains and it was assumed that those chains are capable of rhythmic activity. A similar assumption was put forward by Ranson and Hinsey (1931).

All the chains of neurones found in the central nervous system can be

reduced to two fundamental types (I and II in fig. 3, Lorente de Nó, 1933a) which when combined as shown in I, figure 4 constitute a closed chain, which is thought of being capable of creating a rhythmic series of impulses (closed self-reëxciting chains).

The preparation used in the present experiments is composed of course, of such chains (fig. 4, I); fibres *f* represent the long paths stimulated directly by the shocks through electrodes *F*; neurones *i 1*, and *i 2* represent the nuclei of the reticular substance.

Facilitation may be explained in the following way. The facilitating shock sets up an impulse in fibre *f* which is conducted to the motoneurone *M N* and also to the internuncial neurones *i 1*. If the excitatory process at the motoneurones is subliminal no visible response will appear; but the internuncial neurone *i 1* after the necessary synaptic delay will fire an

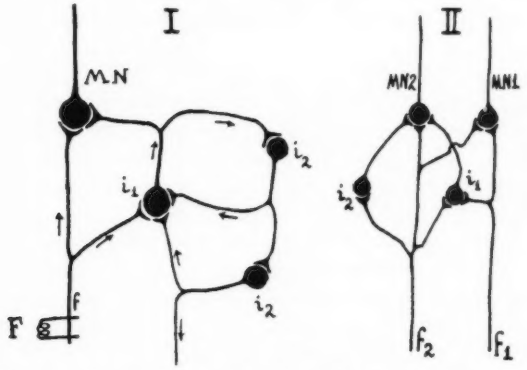


Fig. 4. I. Diagram of a closed self-reëxciting chain of neurones. II. Diagram to explain how the latency of responses is shortened by facilitation.

impulse. If at the moment of arrival of the internuncial impulse at the motoneurone a testing shock is delivered through electrodes *F*, the motoneurone will receive simultaneously the *f* and the *i 1* impulses and therefore will be excited above threshold.

According to this when two successive shocks are delivered through the *F* electrodes the beginning of facilitation depends chiefly on the duration of the synaptic delay of the internuncial neurones *i 1*. Of course if the conditioning shock stimulates all of the *f* fibres, facilitation cannot begin until some of the fibres have recovered; but if the synaptic delay is longer than the absolutely refractory period, facilitation will begin some time after recovery. On the other hand, if the conditioning shock excites only a few of the *f* fibres and the synaptic delay is shorter than their absolutely refractory period, facilitation of the response to the testing shock will be

obtained before the absolutely refractory period of the stimulated fibres is over.

The excitatory process at the synapse is very rapidly dissipated (see later) and therefore cannot set up a repetitive discharge of the neurone; besides, between the stimulated fibres *f* and the motoneurones there are no delay-paths (i. e. unidirectional chains with more than two or three internuncial neurones) able to detain the impulses for more than 2 or 3σ; consequently although chains built like the *a-i 1-M N* one readily explain facilitation lasting for 1.5 to 2σ (curve *6*, fig. 1), for longer periods of facilitation another mechanism has to be postulated.

The existence of chains *i 1-i 2* gives the possibility for long lasting facilitation. The impulses *i 1* excite neurones *i 2* which again excite neurones *i 1*; therefore the motoneurones are submitted to a constant bombardment by excitatory (*i 1*) impulses similar to that postulated by Forbes (1922) and by Eccles and Sherrington (1931b) for the explanation of the reflex after-discharge.

When internuncial chains are set in activity by the conditioning shock the *i 1* impulses are at first few in number; but the cyclic activity of the chains progressively engage new internuncial neurones, thus increasing the amount of facilitation. This would explain the slow ascent of the facilitation curves and the late appearance of the maximum of facilitation.

It was assumed (Lorente de Nó, 1932b, 1934a) that the internuncial chains will cease to work and therefore revert to the original state because slowed recovery during activity will raise the threshold of some links and the circulating impulses will fail to pass through them. The recent observation of Gasser (1935) that nerve fibres when submitted to repetitive stimulation develop a large positive after-potential accompanied by subnormality may explain the late cessation of the cyclic activity without further auxiliary hypothesis.

The assumption that facilitation is due to the activity of internuncial relays can be examined by experiment. Evidently if facilitation is due to internuncial neurones, its temporal course must change, like the after discharge in vestibulo-ocular reflexes (Lorente de Nó, 1928, 1933a), if a part of the internuncial relays are destroyed by suitable operations. As a matter of fact, this is what actually happens.

A very convincing experiment is illustrated by the curves *1* to *5* in figure 1. At the beginning of the experiment when the medulla was intact the conditioning shock was followed by a period of facilitation lasting as usual for from 7σ to 15σ, and having the maximum at a 1.25σ interval between shocks. But later when the vestibular nuclei and the reticular substance in the medulla were destroyed the period of facilitation became considerably lengthened and the maximum of facilitation was found much later, occasionally at a 6σ interval between shocks (fig. 1, curve *4*). This

had to be expected because such a lesion is known to increase the duration of the after-discharge of vestibular reflexes (Lorente de Nó, 1933a, expt. 5).

Another typical experiment is illustrated by curves *1* and *2* in figure 5. In this preparation after extirpation of the cerebellar vermis the vestibular nuclei were destroyed by means of several superficial cuts extending from the level of the abducens nucleus down to near the calamus scriptorius. The preparation was very excitable and showed only slight spontaneous changes of excitability. Only a very slight facilitation was demonstrable having its maximum at an interval between shocks of 1.75σ (curve *1*). Without removing the stimulating electrodes a longitudinal section of the raphe was made extending from the level of the caudal pole of the motor

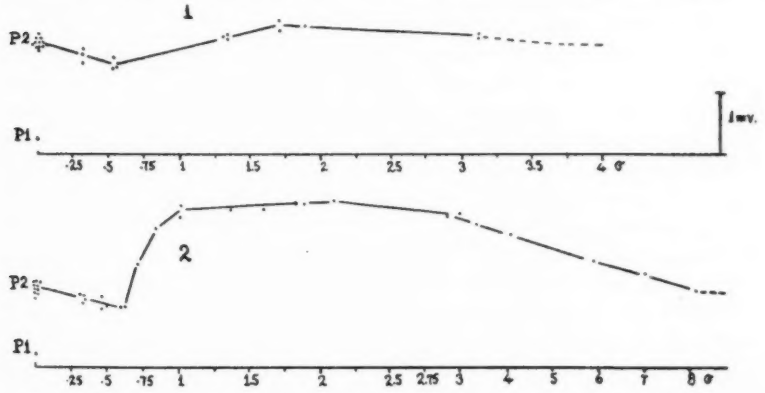


Fig. 5. Potential of the response to the testing *F* shock (ordinates) plotted against the interval in sigmata (abscissae) by which it followed the conditioning *F* shock. *P 1* and *P 2* potentials of the responses to the facilitating and to the unconditioned testing shock. Experiment 11/23/34. 1, after destruction of the vestibular nuclei. 2, after section of the medulla in the middle line.

nucleus of the trigeminus to the level of the oral pole of the nucleus ambiguus. This section is known to produce very radical changes of the vestibular reflexes. (Lorente de Nó, 1928, 1933.) The excitability of the preparation did not change appreciably, so that both the conditioning and the unconditioned testing shocks produced almost equally strong responses as before; but when in succession the testing one set up much stronger responses, the facilitation curve became a typical one. Facilitation lasted for 8σ after the conditioning shock.

Determination of the duration of the excitatory process of the motoneurones created by a volley of impulses. It was indicated in a previous paper (Lorente de Nó, 1935c) that the excitatory process created at the motoneurones by incoming impulses cannot remain at full strength for more

than 0.43σ. This means that it must be dissipated at a fast rate. The experiments reported in this paper seem to prove that the dissipation must be very advanced even at that short interval of time.

For instance, curves *1* and *2* in figure 1 show that when the conditioning shock was very small, the response to the testing one was not greatly modified until the period of facilitation began; but curve *3* shows that when the conditioning shock although still subliminal was larger, the response to the testing one was considerably depressed before it began to be facilitated. The depression started at about 0.25σ interval and was maximum at about 0.5σ.

The importance of this fact made it advisable to repeat the observation as often as possible and therefore the initial period of depression was studied in many experiments (see, for instance, fig. 5). A very well controlled one is illustrated by curve *4* in figure 2. The medulla was not damaged during the extirpation of the cerebellar vermis and no spontaneous changes of excitability were observed; the responses of the motoneurones to a given *F* shock remained constant within a few per cent. The great excitability of the preparation made it possible to use very weak shocks, which when delivered to the oculomotor nerve set up very small responses; in fact the conditioning one was only a few per cent above threshold, so that it could excite only fast *A* fibres.

When delivered through electrodes *F* the conditioning shock was entirely subliminal and the testing one set up a response with potential *P 4*. The heights of the responses to the conditioned testing shock are given in curve *4*, figure 2. Following the conditioning shock it appeared first a period of depression followed by a period of facilitation having the usual characteristics (maximum at 1.25σ, duration about 7σ). Several of the records obtained have been reproduced in figure 3, *15–19*. The relation between the strengths of conditioning and testing shocks was 60/100. This eliminated the effect of post cathodal depression of fibres stimulated subliminally by the conditioning shock. On the other hand depression of the response to the testing shock due to postcathodal depression of nerve fibres should appear earlier and disappear sooner.

Discussion. After considering several possible explanations of the early period of depression, only the following one has been found satisfactory.

The diagram on top of figure 2 includes one of the motoneurones (*M N*) which responded to the unconditioned testing shock. Let it be assumed that four fibres (*f 1–f 4*) had synapses on that motoneurone and that the summation of the excitatory processes in all four synapses was necessary in order to excite the neurone above threshold. The conditioning shock set up impulses only in fibres *f 1* and *f 2*, the testing one in all of them.

When the conditioning shock was delivered fibres *f 1* and *f 2* conducted

impulses which created at their synapses excitatory processes. The testing shock delivered during the absolutely refractory period of fibres *f 1* and *f 2* was able to set up impulses only in fibres *f 3* and *f 4*, but of course the neurone was placed under the influence of the four excitatory processes *f 1–f 4*. As long as *f 1* and *f 2* remained at full value threshold excitation was created, but as soon as *f 1* or *f 2* began to be dissipated the excitation became subliminal.

Since according to curve *4* (fig. 2) the response remained full sized during 0.2σ it has to be concluded that the excitatory process at synapses *f 1* and *f 2* remained at full value for that length of time. The following depression has to be interpreted as a sign of a very fast dissipation of the synaptic excitatory process. However as the internuncial neurones began to fire new excitatory impulses after their synaptic delay, it is impossible to state whether any amount of excitatory process overlasted the absolutely refractory period of fibres *f 1* and *f 2*.

The shortening of the synaptic delay by facilitation. It was reported in a previous paper (1935a) that a long synaptic delay may be progressively reduced to a minimal value of about 0.6σ to 0.7σ by increasing the strength of the stimulus, i.e., by increasing the number of active synapses. However, later experiments have shown that the synaptic delay in each preparation varies only within very narrow limits and that therefore the mentioned reduction might have been due to the fact that strong responses are sooner detectable than weak ones. As a matter of fact when the amplification was arranged so that all responses (weak and strong) were recorded at constant height their latencies became practically identical.

On the other hand, no great significance can be ascribed to slight shortenings of latency produced by increasing the stimulating shock, because stronger shocks may stimulate fibres with shorter conduction times located at relatively great distance from the *F* electrodes.

The synaptic delay in responses consisting of synchronous twitches cannot be appreciably shortened by facilitation. For instance in figure 3 records *15–19* all the responses appear after practically identical latencies, and the same is true for records *1* and *6* in spite of the fact that in record *6* the response is several times larger than in *1*.

However, variations of latency of the response to *F* shocks actually were brought about by facilitation. When the response to the testing shock alone consisted of an asynchronous twitch it was possible to convert it by facilitation into a synchronous one. Records *20* and *21* in figure 3 were obtained in a very well controlled experiment. The conditioning shock was just above threshold and set up a very small response; the testing shock was 250/100 larger and set a response (record *20*) composed of two twitches, the first with a latency of 1.75σ and the second with about 3σ latency (synaptic delays about 0.75σ and 2σ).

When facilitated the testing shock did not excite many more motoneurones than when acting alone, because the response (fig. 3, *21*) consisted of a synchronous twitch with a potential nearly equal to the sum of the potentials of the two previous twitches. But the latency of the response of the motoneurones producing the second twitch was considerably reduced, so that they responded at the same time as those of the first twitch of the unconditioned response. The facilitation consisted only in reducing that latency and it is extremely interesting that at no interval between shocks did a smaller reduction of the latency take place;[5] the facilitated response consisted either of two twitches at the same interval as in the unconditioned one or of a single synchronous twitch. This means that the reduction of the latency is not gradual but steplike, each step being about equal to the known minimal synaptic delay.

A similar case of steplike shortening of the latency of the response is illustrated by records *2*, *3* and *4* in figure 6.

Discussion. The steplike reduction of the synaptic delay, which also was observed in other experiments (Lorente de Nó, 1935a, p. 280) undoubtedly means that the facilitated impulses did not need to pass through an internuncial neurone and thus acted directly on the motor one. Diagram II in figure 4 may help to visualize the happenings in the brain stem. The unconditioned testing shock was producing a response of motoneurone *M N 1* through fibres *f 1* and *f 2* and a response of motoneurone *M N 2* through internuncial neurones *i 1* and *i 2*. Therefore the response of *M N 2* had a synaptic delay twice the delay of *M N 1*. The conditioning shock set up an impulse only in *f 1*, which although subliminal for the motoneurones was able to excite internuncial neurone *i 1* above threshold, thus facilitating motoneurone *M N 2* after the ordinary synaptic delay. At that moment the impulses set up by the testing shock in fibres *f 1* and *f 2* were able to excite both motoneurones above threshold and the response became a synchronous twitch.

The fact that the synaptic delay of the motoneurones is practically invariable and cannot be shortened by facilitation does not agree with the explanation given by Eccles and Sherrington (1931a) for the shortening of latency observed in the spinal flexor reflex; but it has to be considered that the long latencies of the spinal reflexes indicate that several internuncial neurones must be passed by the excitatory impulses. As a matter of fact the records published by Gasser and Graham (1933, fig. 9) show that the delay of the early internuncial potentials is no more than 0.7σ. Since in the reflex arcs long afferent and efferent fibre paths are included,

[5] Since the determination of the start of a potential wave which encroaches upon a preëxisting one can not be made accurately it can not be ruled out conclusively whether the delay was reduced by a few per cent before the steplike shortening took place.

where the impulses must undergo a considerable temporal dispersion a steplike reduction of the latency cannot be detected. On the other hand an error of a few per cent in the measurement of the speed of conduction in the afferent or efferent paths can introduce a considerable error in the calculation of the minimal latency.

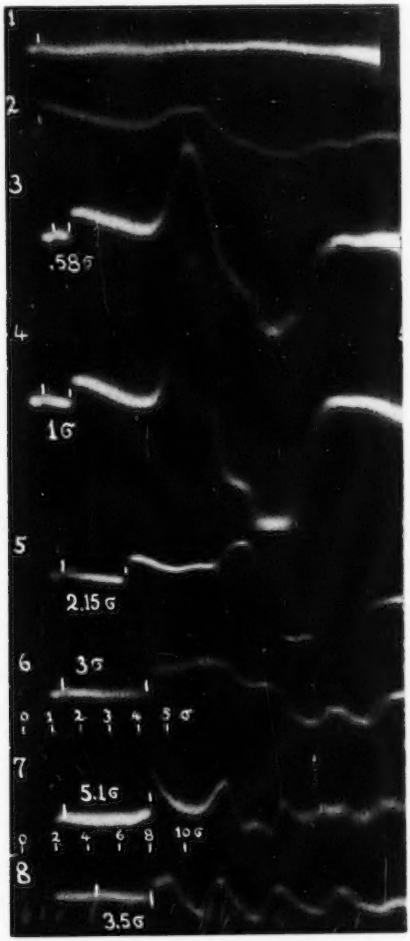


Fig. 6. Experiment 6/19/34. Vestibular nuclei destroyed. Two shocks through the *F* electrodes. Relation between conditioning and testing shock 80/100; *1*, conditioning shock alone; *2*, testing shock alone; *3*, *4*, *5*, *6*, both shocks; *7*, the testing shock is strengthened; *8*, the conditioning shock is made 120/100 of the testing one. Records *1–6* and *7–8* have the same time scales shown in *6* and *7*.

2. *The conditioning shock was followed by a first period of facilitation and a second one during which the testing shock elicited a series of responses.* The experiments reported thus far make it very likely that facilitation is due to the activity of internuncial neurones; the following ones seem to furnish more direct proofs for that assumption.

After a single strong shock through the *F* electrodes one occasionally observes a response composed of a series of twitches. The first twitch appears after the ordinary synaptic delay and is followed at from 5 to 8σ interval by a second much smaller asynchronous twitch, which may be followed after a somewhat longer interval by a third still smaller one. Since the *F* shocks set up only one impulse in each *f* fibre (fig. 4, I) it is evident that the delayed twitches must be due to discharge of internuncial impulses.

Those series of responses are more often seen when two *F* shocks at a short interval are used. The experiment illustrated by the records in figure 6 is typical.

The conditioning shock was subliminal (*1*) but the unconditioned testing one set up a response (*2*) consisting of an asynchronous twitch with an unusually long latency. Undoubtedly the response was being set up through internuncial neurones. When facilitated the testing shock set up a much larger response consisting of a synchronous twitch with a shorter latency. Facilitation began at a 0.56σ interval between shocks (*3*) and was maximal at a 1σ interval (*4*); but it lasted only for a short period of time (no more than 2σ). After that the responses to the testing shock consisted of a series of small twitches at high frequency (*5* and *6*). The number of twitches in the series and their height could be increased by strengthening either the testing shock (*7*) or the conditioning one (*8*).

The high frequency of the twitches excludes the possibility of their having been due to repetitive discharges of the same motoneurones (see the curves in fig. 2 in Lorente de Nó, 1935c); therefore it must be admitted that each twitch was due to different motoneurones, which were excited through paths containing a variable number of internuncial neurones.

The logical explanation of the variation of the type of response to the testing shock with the increase of the interval by which it was preceded by the conditioning one is that the activity of the internuncial relays changed progressively. Immediately after the conditioning shock the internuncial impulses were conducted to the motoneurones, which thus were being excited subliminally; the testing shock was therefore able to set up a larger response; but later the internuncial impulses went through other paths and did not reach the motoneurones. Consequently, the testing shock did not set up any larger response than usual (compare records *5* and *6* with record *2*) but reënforced the activity of the internuncial chains, which began setting up volleys of impulses, each one composed of a number

of impulses high enough to reach the threshold of some motoneurones. Judging by the duration of the series of twitches, the activity of the internuncial relays was increased during 20σ to 30σ.

Discussion. The ability of the conditioning shock to start a process by virtue of which the motoneurones receive volleys of internuncial impulses, seems to lie beyond reasonable doubt. But of course, it still has to be demonstrated that internuncial impulses actually arrive at the motor nucleus during the period of facilitation, which follows a conditioning shock.

Two different proofs can be offered. On one hand it has been determined that the "tonic" waves in the electrogram of the muscle increase during the period of facilitation and besides internuncial potentials have been recorded from the pathways entering the motor nucleus.

Records *10* and *11* in figure 3 show that after a weak conditioning twitch (*t*) the tonic waves were not greatly modified (*10*) but after a larger twitch (*11*) they increased considerably during a period of time which closely corresponded to the duration of the period of facilitation of the response to the testing shock. Unfortunately the effect of the conditioning shock on the tonic innervation has not been examined systematically in all the experiments. But in the six cases in which observations were made (Lorente de Nó, 1935e) the results were concordant.

To record the potentials developed by the impulses which arrive at the motor nucleus involves a very difficult technical problem, because both the stimulating and the recording electrodes have to be placed on the brain stem only a few millimeters apart, so that the escape of the stimulating shock is many times larger than the potentials to be recorded. After many unsuccessful trials in which the shock artefact threw the amplifier tubes off of the characteristic, satisfactory results were obtained in two experiments.

Figure 3 contains two of the records (*8* and *9*) obtained in one experiment arranged as follows. The stimulating electrodes were placed as usual on the floor of the fourth ventricle and the responses of the internal rectus muscle were recorded (fig. 1, curves *1* to *5*; fig. 3, records *1* to *7*). Without removing the stimulating electrodes the rabbit was decerebrated through a section oral to the anterior colliculus; besides the roof of the colliculi together with the oculomotor nuclei were extirpated by means of an oblique section with a sharp knife. The ground electrode was placed on the zone where the paths converge towards the oculomotor nucleus and the grid one some 4 mm. distant on the cut surface of the colliculus.

Record *8* (fig. 3) obtained with subliminal shocks contains only shock artefacts (*a*, *a*) but in record *9* following each artefact (*a*) there appear two potential waves (*w 1* and *w 2*). The first wave (*w 1*) undoubtedly signals the arrival to the nucleus of the impulses set up by the stimulating shock

in fibres *f* (fig. 4, *I*) and their branches. Its long duration indicates that there is a considerable temporal dispersion of the impulses, which, since the distance of conduction is very short, has to be attributed to branching of the *f* fibres. The second wave (*w 2*) must have been produced by internuncial impulses; it also is asynchronous and its crest coincides with the maximum of facilitation in curves *2* and *3*, figure 1.

The amplification used in obtaining records *8* and *9* was the highest compatible with a relatively undisturbed base line but it was of course very low. When it was increased the whole interval between waves *w 1* and *w 2* was filled with smaller potential waves; other small waves appeared after *w 2*.

In closing this discussion it may be said that although the technical difficulties made the observations not as exact as it should be desired, it can be considered as an established fact that a single shock through the *F* electrodes causes the arriving to the motor nucleus of waves of negative potential during the whole period of facilitation, the maximum of potential corresponding to the maximum of facilitation.

SUMMARY

The experimental material collected in this paper is considered as evidence that:

1. The excitatory process of the motoneurones created by a volley of impulses has a very short duration; it does not remain at full value for longer than 0.2 to 0.25σ and is dissipated very rapidly. It has not been possible to ascertain whether it is entirely destroyed before the stimulated fibres recover from absolute refractoriness.

2. Facilitation of the motoneurones is due to the arrival to the motor nucleus of internuncial impulses.

3. The internuncial neurones are arranged in closed self-reëxciting chains (reverberating chains of Ranson and Hinsey) capable of sustained rhythmic activity.

4. The synaptic delay of the motoneurones is a fixed quantity which cannot be appreciably reduced by facilitation.

5. The shortening of the latent period of reflexes by facilitation is due to the fact that when facilitated the excitatory impulses do not need to be reënforced by passage through internuncial neurones.

6. Experimental proofs of the arrival of internuncial impulses to the motor nucleus during the period of facilitation are given.

REFERENCES

CREED, R. S., D. DENNY BROWN, J. C. ECCLES, E. G. T. LIDDELL AND C. S. SHERRINGTON. Reflex activity of the spinal cord. Oxford, 1932.

DENNY BROWN, D. AND C. S. SHERRINGTON. J. Physiol. **66**: 175, 1928.

DUSSER DE BARENNE, J. G. AND A. DE KLEYN. Acta otol. **16**: 97, 1931.

ECCLES, J. C. AND C. S. SHERRINGTON. Proc. Roy. Soc. B **107:** 511, 1931a; Proc. Roy. Soc. B **107:** 586, 1931b.

FORBES, A. Physiol. Reviews **2:** 361, 1922.
The mechanism of reaction, in A handbook of general experimental psychology. Worcester, Mass. p. 155, 1934.

FULTON, J. F. Muscular contraction and the reflex control of movement. Baltimore, Md., 1926.

GASSER, H. S. This Journal **111:** 35, 1935.

GASSER, H. S. AND H. T. GRAHAM. This Journal **103:** 303, 1933.

HUGHES, J. AND H. S. GASSER. This Journal **108:** 295, 1934a. This Journal **108:** 307, 1934b.

KÖLLNER, H. AND P. HOFFMANN. Arch. Augenheilk. **90:** 170, 1922.

LORENTE DE NÓ, R. Trav. Lab. Rech. Biol. Madrid **23:** 295, 1925.
Die Labyrinthreflexe auf die Augenmuskeln, etc. Wien 1928.
Ergebn. Physiol. **32:** 73, 1931.
The Laryngoscope **42:** 234, 1932a.
Trans. Am. Otol. Soc. May, 1932b.
Arch. Neurol. and Psych. **30:** 245, 1933a.
The Laryngoscope **43:** 327, 1933b.
J. f. Psychol u. Neurol. **45:** 381, 1934a.
J. f. Psychol u. Neurol. **46:** 113, 1934b.
This Journal **111:** 272, 1935a.
This Journal **111:** 283, 1935b.
This Journal **112:** 595, 1935c.
Acta Otol. **21:** 416, 1935d.
Inhibition of motoneurones, to be published in the Symposium in honour of Prof. Beritoff, 1935e.

RANSON, S. W. AND J. C. HINSEY. This Journal **94:** 472, 1930.

SHERRINGTON, C. S. Proc. Roy. Soc. B **97:** 519, 1925.
Proc. Roy. Soc. B **105:** 332, 1929.

SPIEGEL, A. Ztschr. Hals-usw. Heilk. **25:** 200, 1929.

THE SUMMATION OF IMPULSES TRANSMITTED[1] TO THE MOTONEURONES THROUGH DIFFERENT SYNAPSES

R. LORENTE DE NÓ

From the Central Institute for the Deaf, St. Louis, Mo.

Received for publication June 3, 1935

The modifications of the response of the motoneurones to a given synaptic stimulus brought about by a preceding stimulation were described in a recent paper (Lorente de Nó, 1935d); however, there was a complicating factor in those experiments, namely, that the conditioning and the testing impulses were set up in the same pathways. The present experiments were planned to eliminate that complication.

TECHNIQUE. The preparation was described in previous papers (1935a, b), but in order to set up impulses in different pathways two pairs of electrodes were used, each connected with a different induction coil.

One pair of electrodes (*F*) was placed as usual on the floor of the fourth ventricle, on the left abducens nucleus, and the other pair (*C*) was introduced in the right superior colliculus, so that their free points reached the reticular nuclei in front of the nucleus ruber, which are known to send fibres to the oculomotor nuclei. As usual the responses of the left internal rectus muscle were recorded with a cathode ray oscillograph.

RESULTS. A shock through the *F* electrodes modifies the response to a following shock through the *C* ones and conversely. The results obtained are to a large extent the same as when a first shock through the *F* electrodes modifies the response to a second shock through the same electrodes (Lorente de Nó, 1935d). The results obtained will be described on hand of curves *1–4* of figure 1.

In the case of curve *1* the subliminal *C* shock preceded the *F* one, which when alone set up a very small response after the ordinary synaptic delay. When preceded by the *C* one the *F* shock set up much larger twitches; the earliest facilitated response was seen at a 0.25σ interval between shocks, the twitches increased gradually with the shock interval and attained their greatest size at a 1σ interval, later the facilitation decreased and was not demonstrable at shock intervals of more than 7σ.

In the case of curve *2* the *C* shock preceded the *F* one, which, when unconditioned, set up after the ordinary synaptic delay a response with

[1] The work reported in this paper has been aided by a grant from the Rockefeller Foundation.

potential $P f$. The C shock was just above threshold and set up a scarcely visible response.

When both shocks were delivered simultaneously the response to the F one was much greater than the unconditioned one. The facilitation diminished rapidly when the interval between shocks was increased, and at a 0.5 to 0.6σ interval no facilitation was demonstrable; but it reappeared

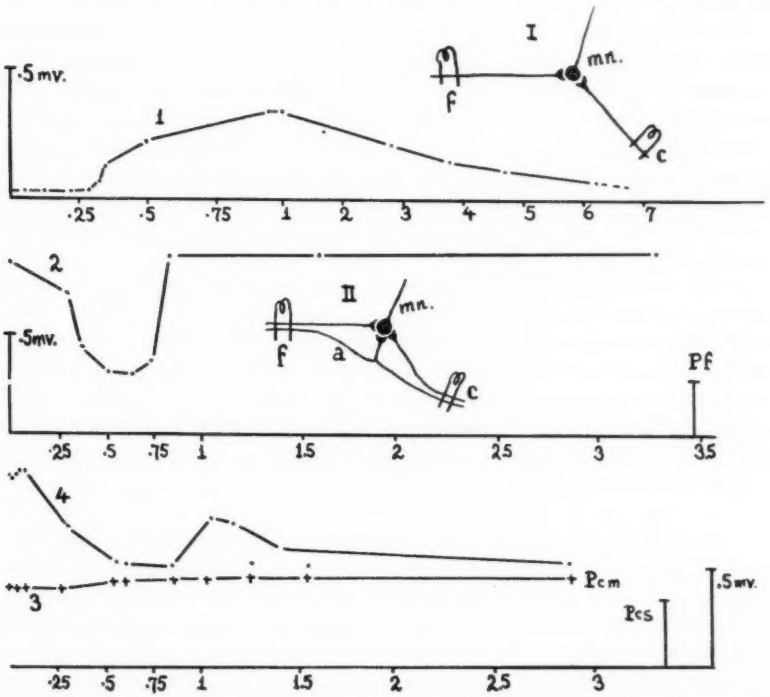


Fig. 1. Potential of the response to the testing shock (ordinates) plotted against the interval in σ by which it was preceded by the conditioning shock (abscissae).

1. Experiment 29/12/34. The conditioning C shock is subliminal. Diagram *1* indicates that each shock excited different fibres. *mn.*—motor nucleus; *f.* 1, *c.*—stimulating electrodes.

2. Experiment 7/1/35. The subliminal C shock is the conditioning one. *Pf*, potential of the response to the unconditioned floor shock.

3, 4. Experiment 7/1/35. The subliminal F shock is the conditioning one; curve *3* is a plot of the potential ($P\,c\,m$) of the motor component of the C response; the lack of facilitation indicates that it was due to direct excitation of some fibres of the oculomotor nerve; curve *4* is a plot of the potential of the synaptic C response; $P\,c\,s$.—potential of the synaptic component of the unconditioned C response. Diagram II indicates that in the cases of curves *2*, *3* and *4* a number of fibres *a* were excited by both shocks. (See fig. 1 in Lorente de Nó, 1935a.)

at a shock interval of about 0.8σ and ceased about 7σ after delivery of the C shock.

In the case of curves *3* and *4* a subliminal F shock was the conditioning one; the testing C shock when unconditioned set up a response consisting of two twitches, the first had the latency of a motor twitch and the second one, with potential $P\ c\ s$ had a longer latency, which included the synaptic delay of the motoneurones.

The motor twitch was not appreciably modified by the preceding F shock (curve 3) but the synaptic response was facilitated at the intervals between shocks indicated by curve *4*. Facilitation was greatest at simultaneity, it decreased rapidly when the shocks were separated and became minimal at intervals from 0.5 to 0.75σ; later it increased again and showed a second maximum when the testing C shock followed the conditioning F one by 1σ. Slight facilitation was still demonstrable at a 7σ interval.

Discussion. The interpretation of curves *1*, *2* and *4* is not difficult in view of the following facts:

1. The strength of the excitatory process of the motoneurones (measured by its ability to break through the relative refractoriness created by an antidromic impulse) is roughly proportional to the number of activated synapses (Lorente de Nó, 1935c); and it is reasonable to assume that when the number of active motoneurones of the motor nucleus increases the excitation of the individual motoneurones is stronger (Sherrington, 1931).

2. The excitatory process created by an impulse at the synapse is very rapidly dissipated and does not remain at full value for longer than 0.2 to 0.25σ (Lorente de Nó, 1935d).

3. A shock delivered to paths entering the nerve centres (posterior roots of the spinal cord, Gasser and Graham, 1933; Hughes and Gasser, 1934) or to secondary central paths (Lorente de Nó, 1935d) sets up a volley of impulses in those fibres, followed by volleys of internuncial impulses.

On the basis of these facts and since the testing shock sets up a constant volley of impulses, curve *1* has to be interpreted as an indicator of the statistical distribution of the subliminal states of excitation of the motoneurones created by the impulses due to the conditioning shock. To a large extent the ordinates of the curve measure the number of facilitating impulses arriving at the motor nucleus at the corresponding interval after the conditioning shock. Temporal dispersion in fibre paths and later in paths including internuncial neurones are the main factors in the determination of the shape of the curve.

Curve *1*, however, does not measure the total number of facilitating impulses entering the motor nucleus, because it begins to ascend about 0.25σ after delivery of the conditioning shock, while there is no doubt that the latter set up an initial volley of impulses, without appreciable latency,

which as curves *2* and *4* show may produce a great facilitation of the motoneurones. Therefore what the ordinates of curve *1* measure is the number of impulses which when summated with the testing ones actually bring motoneurones into threshold excitatory state.

After considering several possible explanations of the gap in curves *2* and *4* at 0.5 to 0.8σ interval between shocks it has been found that the only satisfactory one is the assumption that the conditioning and the testing shocks in part stimulated the same fibres (*a* in diagram II, fig. 1). This assumption is based upon the fact that a number of the secondary and reticular paths give only collateral branches to the oculomotor nuclei (fibres *c* and *d* in Lorente de Nó, 1935a, fig. 1). While those fibres were refractory, the number of testing impulses was actually reduced and therefore only a relatively small number of motoneurones reached threshold excitation. After recovery facilitation of the testing response was again produced. Curves *2* and *4* are thus the resultant of two curves, one measuring the temporal course of dissipation of the excitatory process created by the initial volley of conditioning impulses, and the other, similar to curve *1*, but modified by refractoriness of fibres, measuring the number of delayed facilitatory impulses. The facilitation curves obtained with two *F* shocks, the first of which was the smallest (Lorente de Nó, 1935d) have to be interpreted in a similar way, only that because the conditioning impulses were conducted through the same paths as the testing ones the initial period of summation did not appear; evidently both shocks in quick succession could not produce any higher number of impulses than the testing shock alone.

The fact that in curves *2* and *4* the responses were maximal when both shocks were delivered simultaneously and rapidly diminished when the interval between shocks was made longer than 0.15σ to 0.20σ indicates that a condition for optimal summation of impulses on the motoneurone is simultaneity of arrival. However, the lack of facilitation in the initial part of curve *1* and the fact that a volley composed of a great number of impulses set up by an *F* shock remains ineffective, if there is no previous background of excitation (Lorente de Nó, 1935d), indicates that for effective summation of impulses still other conditions must be fulfilled.

The short duration of the period of effective summation of two nerve impulses (0.15 to 0.20σ) explains why the synaptic delay of the motoneurones is practically invariable (Lorente de Nó, 1935d). The shortening brought about by facilitation cannot be greater than the duration of the period of summation of the impulses and although shortenings of that order of magnitude were observed, it could not be excluded that they were due to imperfections in technique. It is intended now to repeat the observations leading the action potentials of the response from the oculomotor nerve itself, so that variations of latency of 0.1σ or less will be measurable with sufficient accuracy.

CONCLUSIONS

The condition for optimal summation of excitatory impulses is simultaneity of arrival at the motoneurones. A delay of more than 0.1 to 0.2σ greatly reduces their summated excitatory value.

The curves of temporal course of facilitation presented in this and in a preceding paper (Lorente de Nó, 1935d) are interpreted as the resultants of two different curves, one measuring the rate of dissipation of the excitatory process created by the initial volley of facilitating impulses and the other roughly indicating the temporal course of the arrival to the motor nucleus of delayed facilitating impulses.

REFERENCES

GASSER, H. S. AND H. T. GRAHAM. This Journal **103:** 303, 1932.
HUGHES, J. AND H. S. GASSER. This Journal **108:** 295, 307, 1934.
LORENTE DE NÓ, R. This Journal **111:** 272, 1935a. This Journal **111:** 283, 1935b. This Journal **112:** 595, 1935c. This Journal **113:** 505, 1935d.
SHERRINGTON, C. S. Brain **54:** 1, 1931.

SURVIVAL AND INCREASE OF EPINEPHRINE IN TISSUE CULTURES OF ADRENAL GLANDS FROM CHICK EMBRYOS

MARGARET REED LEWIS AND EUGENE M. K. GEILING

From the Department of Embryology, Carnegie Institution of Washington, and Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University, School of Medicine

Received for publication June 21, 1935

Tissue cultures offer an effective method for the study of some of the fundamental problems connected with the endocrine glands, especially those of the adrenals. Although little or no chromaffin is present in embryos of 5 to 6 days' development, this material becomes stored up in the cultures prepared from the adrenals of chick embryos of this age.

Investigators differ in opinion as to when it is first possible to demonstrate the presence of epinephrine in the chick embryo. Lutz and Case (1925), by using enucleated eyes of frogs as test material, were able to show epinephrine in chick embryos as early as the eighth day of incubation. Okuda (1928) found the amount of adrenalin in standard content to be less than 0.0001 mgm. in chick embryos of 9 days' growth. By using 10 to 20 embryos for one extract he could detect adrenalin after 8 days but no positive test was secured from those of 7 days' growth. On the other hand, Hogben and Crew (1923) obtained only slight and even doubtful positive tests on strips of cat's ileum with extracts of adrenals from 5 chick embryos of 14 days' incubation, but extracts of embryos of 16, 19 and 20 days' development gave good results.

In 1926 E. H. Miller was able to demonstrate by means of smooth muscle reactions that the amount of chromaffin present in sections of embryonic adrenals corresponded roughly with the amount of epinephrine present in the adrenals of developing embryos. It was therefore determined to ascertain whether the chromaffin, differentiated in tissue cultures of adrenal glands and fixed in solutions containing chromic salts (Zenker's solution), indicated the presence of epinephrine and whether, with the increase of chromaffin that takes place in the tissue cultures, there occurred a corresponding increase in epinephrine.

METHOD. Tissue cultures were prepared in the usual manner—small pieces of adrenal gland, taken from chick embryos, were explanted into hanging drops either of different hydrogen-ion nutrient saline concentrations or of chicken plasma and allowed to grow for 3 days. In the greater number of experiments carried out in this study the chick embryos used

were of 6 and 7 days' growth, and, in most instances, the adrenals from 10 or 12 chick embryos were used in preparing cultures for one test. Materials for comparison of the epinephrine content of the cultures were extracted from the adrenals of chick embryos that had been incubated for the same length of time (6–7 days) as those used for the cultures and also from the adrenals of embryos (9–10 days old) that had been permitted to continue in the incubator for the length of time the explanted adrenal tissue had grown in the cultures.

A few experiments were carried out in which cultures were made from embryos of various stages of development (5–14 days). The epinephrine content of these cultures was compared with that of fresh adrenals from embryos of corresponding ages of development and with standard epinephrine solution (1:100,000).

After the cultures had grown 3 days the tissue was removed and ground in a few drops of 0.25 per cent acetic acid. Sufficient acetic acid was added to bring the total volume to about 2 cc. The suspension was then transferred to a graduated pyrex centrifuge tube, heated for five minutes

---

Chart 1. Blood pressure action on cats under luminal anesthesia following injections of extracts of chick embryo adrenals ground in 0.25 per cent acetic acid. The total volume was made up proportional to the number of embryos used, heated and centrifugalized. The injections were made of extracts as follows:

Cat 1. Weight 2.85 kgm.

Fig. 1. 0.5 cc. of extract of adrenals from 5 embryos 6 days old.
Fig. 2. 0.5 cc. of extract of adrenals of 5 embryos 6 days old grown 3 days in tissue cultures.
Fig. 3. 0.1 cc. of 1–100,000 solution of standard epinephrine.
Fig. 4. 0.2 cc. of 1–100,000 solution of standard epinephrine.

Cat 2. Weight 3.45 kgm.

Fig. 1. 0.2 cc. of 1–100,000 solution of adrenalin.
Fig. 2. 0.2 cc. of extract of adrenals of 12 chick embryos 7 days old grown 3 days in tissue cultures.
Fig. 3. 0.3 cc. of same extract as figure 2.
Fig. 4. 0.4 cc. of same extract as figure 2.
Fig. 5. 0.2 cc. of 1–100,000 solution of adrenalin.

Cat 3. Weight 3.2 kgm.

Fig. 1. 0.2 cc. of 1–100,000 solution of epinephrine.
Fig. 2. 0.2 cc. of extract of adrenals of 10 chick embryos 7 days old.
Fig. 3. 0.2 cc. of extract of adrenals of 12 chick embryos 7 days old grown 3 days in tissue cultures.
Fig. 4. 0.2 cc. of extract of adrenals of 10 chick embryos 10 days old.
Fig. 5. 0.2 cc. of the culture medium from tissue cultures of chick embryo adrenals.
Fig. 6. 0.4 cc. of 1–100,000 solution of epinephrine.
Fig. 7. 0 2 cc. of same extract as injection 2.
Fig. 8. 0.2 cc. of same extract as injection 3.
Fig. 9. 0.2 cc. of same extract as injection 4.

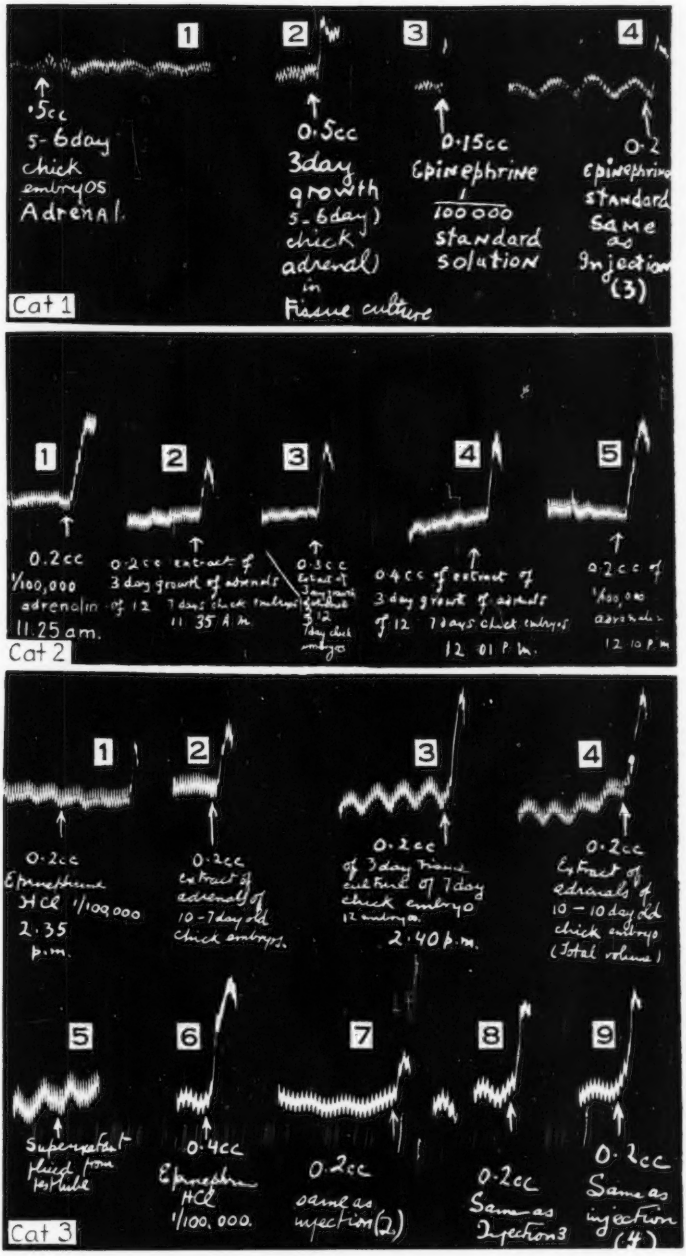

1
2
3
4
.5cc
5-6day
chick
embryos
Adrenal.
0.5cc
3day
growth
5-6day)
chick
adrenal)
in
Tissue culture
0.15cc
Epinephrine
1/100000
standard
solution
0.2
epinephrine
standard
same
as
Injection
(3)
Cat 1
1
2
3
4
5
0.2cc
1/100,000
adrenalin
11.25 a.m.
0.2cc extract of
3 day growth of adrenals
of 12 7 days chick embryos
11 35 A.M.
0.3cc
Extract of
3 day growth
of 12
7 day chick
embryos
0.4cc of extract of
3 day growth of adrenals
of 12 7 days chick embryos
12 01 P.M.
0.2cc of
1/100,000
adrenalin
12 10 P.M.
Cat 2
1
2
3
4
0.2cc
Epinephrine
HCl 1/100,000
2.35
p.m.
0.2cc
extract of
adrenals of
10-7 day old
chick embryos.
0.2cc
of 3 day tissue
culture of 7 day
chick embryo
12 embryos.
2.40 p.m.
0.2cc
Extract of
adrenals of
10 – 10 day old
chick embryo
(Total volume)
5
6
7
8
9
Supernatant
fluid from
test tube
0.4cc
Epinephrine
HCl
1/100,000.
0.2cc
same as
injection (2.)
0.2cc
Same as
Injection 3
0.2cc
Same as
injection
(4)
Cat 3

Chart 1

in a water-bath at about 60°C. and then centrifugalized. The clear supernatant fluid was used for injection and chemical testing.

The presence of epinephrine was demonstrated by 1, blood-pressure changes elicited in anesthetized cats as recorded by an ordinary mercury manometer; 2, dilatation of the pupil in frogs and 3, the ferric chloride reaction.

The blood-pressure action of the tissue cultures and of the embryos was in each instance compared with that of a standard epinephrine solution (1:100,000) (cat 1, figs. 3 and 4; cat 2, figs. 1 and 5; cat 3, figs. 1 and 6).

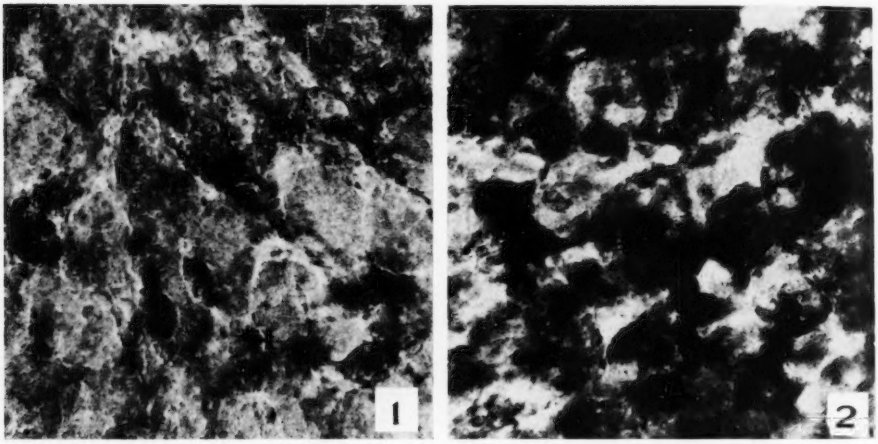


Plate 1. Fig. 1. Photograph of a fixed and stained culture of the suprarenal gland of a 7-day chick embryo grown in nutrient saline 24 hours. The black spots show the brown chromaffin material stored in the cells of the medulla. ×200.

Fig. 2. Photograph of a fixed and stained culture from the same suprarenal gland as that used in figure 1, but permitted to grow in a nutrient medium for three days. This shows an increase in amount of chromaffin in the cells as well as an increase in the number of chromaffin bearing cells. ×200.

Results. In general it was found that chick embryos less than 7 days old had little effect on blood-pressure (chart 1, cat 1, fig. 1). In some instances those of 7 days gave a good response (cat 3, fig. 2) while in others the results were indefinite. The 3-day culture of tissue showed a definite increase in the amount of epinephrine (cat 1, fig. 2; cat 3, figs. 1, 2, 7 and 8). This increase was noted even in some cultures made from embryos (5–6 days) of an age at which stained sections showed no chromaffin material (see chart 1, cat 1, figs. 1 and 2).

The epinephrine content of cultures grown for three days was about equal to that of the adrenals of embryos left in the incubator for the

same length of time (cat 3, figs. 2, 3, 4, 7, 8 and 9). The nutrient medium showed no discernible amount of epinephrine either before or after the growth of the cultures (cat 3, fig. 5).

Injections of increasing amounts of the extracts showed a corresponding increase in action on the blood-pressure of the cat (cat 2, figs. 2, 3, 4 and 5).

In embryos at different stages of development (5–14 days) there occurred progressive increases in epinephrine content. Allowing for variations in the embryos it can be said that in the range with which we worked there was a five-fold increase in epinephrine content in adrenals grown in tissue cultures for a period of three days.

Cultures fixed and stained after 24 hours' growth showed decidedly less chromaffin material (plate 1, fig. 1) than those of the same set fixed after 3 days' growth (plate 1, fig. 2). This corresponds roughly to the increase in epinephrine content during the growth in cultures as discussed above.

We feel justified in stating that the material responsible for the increase of blood pressure in the experiments was epinephrine because we obtained a positive ferric chloride test, because the material was destroyed by alkali and gave similar results upon repeated injection and because after injection into the ventral lymph sac a definite dilatation of the frog's pupil took place.

REFERENCES

HOGBEN, L. T. AND F. A. E. CREW. Brit. J. Exp. Biol. **1**: 1, 1923.
LUTZ, B. R. AND M. A. CASE. This Journal **73**: 670, 1925.
MILLER, E. H. This Journal **75**: 276, 1926.
OKUDA, M. Endocrinol. **12**: 342, 1928.

FURTHER INFORMATION REGARDING THE MELANOPHORE HORMONE OF THE HYPOPHYSIS CEREBRI

EUGENE M. K. GEILING AND MARGARET REED LEWIS

From the Department of Pharmacology and Experimental Therapeutics, Johns Hopkins Medical School, and the Department of Embryology, Carnegie Institution of Washington

Received for publication June 21, 1935

The luxuriant growth of the hypophysis cerebri of adult mammals which takes place in tissue cultures suggested the utilization of this method as a means of obtaining further evidence regarding the site of formation of the multiple principles ascribed to the various lobes of the pituitary gland.

An extensive survey of the hypophyses of readily available mammals (pig, guinea pig, cat, rabbit, rat and mouse) led us to the conclusion that the gland of the mouse (fig. 1) was best suited for obtaining tissue for cultural purposes. The mouse was chosen because it was possible for us to separate most of the intermediate lobe from the pars nervosa as may be seen in the section shown in figure 2. The pituitary gland of the rat (fig. 3) was also suitable for our purposes although, as is shown in the section (fig. 4), owing to the invasion of the neural lobe by the epithelial tissue of the intermediate lobe all of the intermediate lobe could not be freed from the neural lobe.

METHOD. The cultures were prepared in rotating pyrex test-tubes according to the W. H. Lewis (1935) simplification of the G. O. Gey (1933) roller-tube technique. The lobes of the hypophyses were divided into small pieces which were fastened to the inner surface of the tube by means of a thin layer of chicken plasma and bathed by 1 cc. of nutrient medium composed of 7 parts of saline, 2 parts of chick-embryo extract and 4 parts of horse serum. The nutrient solution was changed every four days.

As much of the pars intermedia as possible was separated from the pars nervosa and explanted into one tube; the neural lobe, with a fringe of adhering intermediate lobe, was explanted into a second tube and the fragments of the pars anterior were placed in a third tube.

Cultures of connective tissue were prepared with known amounts of standard pituitary solution added to the nutrient medium in order to determine whether the hormone could survive apart from its specific cells. These cultures were allowed to grow for periods varying from three to fifty days. The tissue was then removed and ground with 0.25 per cent

acetic acid to make a volume of 2 cc. This suspension was transferred to graduated pyrex centrifuge tubes, brought to a boil for half a minute and then centrifugalized. The clear supernatant fluid was used for test-

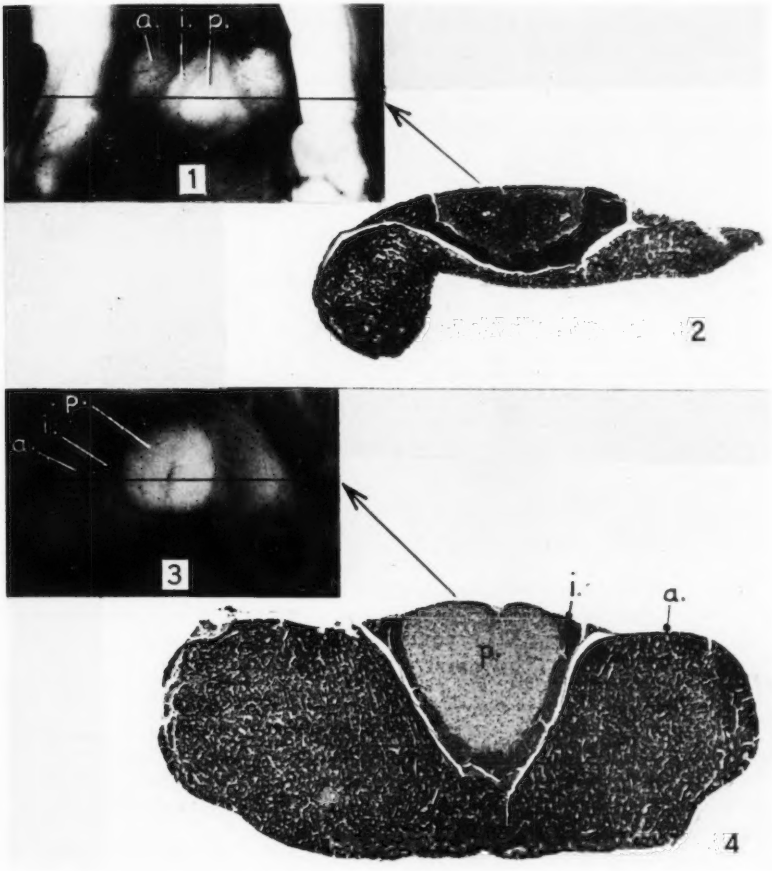


Fig. 1. *A* = pars anterior; *P* = pars nervosa; *I* = pars intermedia.

1. Photograph of the total unfixed mouse hypophysis lying on the floor of the skull. It was exposed through severing the delicate infundibular process. ×10.

2. Photograph of a section of a mouse hypophysis cut in a plane as indicated by the arrow in figure 1. ×30.

3. Photograph of the total unfixed rat hypophysis taken in the same position as that of the mouse shown in figure 1. ×10.

4. Photograph of a section of a rat hypophysis cut in a plane as indicated by the arrow in figure 3. ×30.

ing. The nutrient medium of the cultures was prepared in the same manner for testing.

The material was tested first for its effect on an anesthetized cat (prepared for recording changes in blood-pressure, and second for its melanophore-expanding action on frogs.

These experiments were repeated many times using both the rat and the mouse hypophyses.

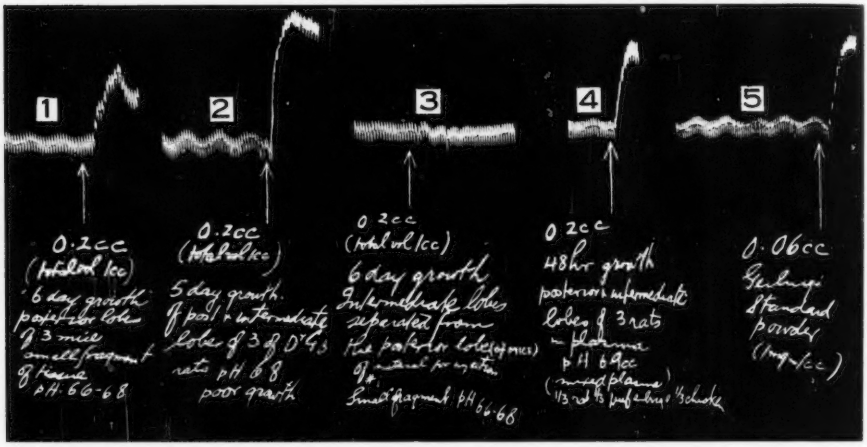


Chart 1. Blood-pressure readings on a cat weighing 3 kgm. under sodium luminal anesthesia. The growths of tissue in the cultures to be tested were ground in acetic acid 0.25 per cent, centrifugalized and 0.2 cc. of each supernatant fluid used for injection. The tissue cultures were as follows:

Fig. 1. The pars nervosa of three mice grown for 6 days in plasma adjusted to pH 6.6–6.8.

Fig. 2. The pars nervosa together with the pars intermedia of three rats grown for 5 days in plasma adjusted to pH 6.8.

Fig. 3. The pars intermedia separated from the pars nervosa (the growth of this was used for injection 1) of three mice grown for 6 days in plasma adjusted to pH 6.6–6.8.

Fig. 4. The pars nervosa and the pars intermedia of 3 rats grown for 2 days in mixed plasma pH 6.9.

Fig. 5. Injection of 0.06 cc. of a solution (1 mgm. to 1 cc.) of Geiling's standard powder of the posterior lobe.

Results. The conclusions arrived at from this study were as follows:

1. The tissue of the different lobes of the hypophyses of the various mammals studied grew luxuriantly in the cultures and in all probability would have remained in good condition much longer than the fifty days required for these observations. In the cultures permitted to grow for two weeks or longer the area of new growth was several times as extensive

as that of the original tissue. The pars intermedia grew as extensive folded membranes of epithelial cells with some growth of radiating stroma cells. The pars nervosa, which in sections appeared to be largely non-cellular, gave rise to an abundant growth of long, flat, radiating cells, many macrophages and extensive networks of large cells with long branched processes resembling neuroglia cells. The growth from the pars anterior took place mainly in the form of wide, flat membranes with some radiating growths of endothelium and other stroma cells. In these membranes it was possible to distinguish three types of epithelial cells. The first, which formed most of the membrane, was of the type common to epithelial growth in cultures. Scattered here and there in groups in the membranes or, in some instances, forming separate small membranes was a second type of cell which contained large irregular albumen granules. The third type of epithelial cell, usually located within the thicker part of the growth or scattered near the explant, had its cytoplasm closely packed with small, brightly-refractive granules (identified in stained preparation as the eosinophilic or chromophil cells).

2. The material derived from the cultures of the pars intermedia possessed no blood-pressure-raising property, but had a marked melanophore-expanding effect when injected into frogs. Injection no. 3 of chart 1 illustrates this point.

3. The cultures of the anterior lobe had no blood-pressure-raising effect nor any melanophore-expanding action when injected into frogs.

4. The cultures of the neural lobe admixed with a ragged edge of pars intermedia had both pressure and melanophore-expanding actions.

5. The cultures of connective tissue, to which the posterior pituitary solution was added, had no pressor action indicating that the hormone had been inactivated.

6. In no instance did the nutrient medium show any appreciable effect on the blood-pressure or on the frogs.

CONCLUSION

The results obtained in this study of tissue cultures furnish not only direct confirmatory evidence in support of the view that the pars intermedia elaborates the melanophore-expanding hormone, but further indicate that the pressor hormone of the so-called posterior lobe of the hypophysis is not formed by the pars intermedia.

REFERENCES

GEY, G. O. Am. J. Cancer **17**: 752, 1933.
LEWIS, W. H. Contributions to Embryology, no. 150, 1935.
ZONDEK, B. J. A. M. A. **104**: 636, 1935.

THE EFFECT OF ULTRAVIOLET RADIATION ON LENS PROTEIN IN THE PRESENCE OF SALTS AND THE RELATION OF RADIATION TO INDUSTRIAL AND SENILE CATARACT

JANET HOWELL CLARK

From the Department of Physiological Hygiene, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

Received for publication June 13, 1935

Burge (1) (2) made the important observation that lens protein will precipitate if radiated with ultraviolet light in the presence of dilute calcium salts (0.1 per cent $CaCl_2$), or if dilute calcium salts are subsequently added to radiated lens protein. He compared the action of the calcium solutions to the action of the developer on an exposed photographic plate and on the basis of his results he proposed the theory that cataracts are due to the action of ultraviolet radiation when abnormal amounts of calcium salts or silicates are present in the lens. He supported this theory by analyses of cataract lenses and normal lenses which showed that cataract lenses contain more calcium, or calcium and silicon, and less potassium than normal lenses.

Burge's conclusions have been criticized by Adams (3) who found only slight opalescence in lenses immersed in calcium chloride solutions and radiated for 3 hours. These lenses, however, were kept at a temperature of 15°C., a temperature too low to favor any marked coagulation of radiated proteins (4).

A recent investigation (4) has shown that coagulation of isoelectric salt-free egg albumin by ultraviolet radiation occurs in three steps. The first step, the light-denaturation of the protein molecule, has a temperature coefficient of 1 and occurs when the protein is radiated at 4°C. but is not followed by flocculation until the radiated protein is heated to a moderate temperature. The second step is a reaction between the light-denatured molecule and water with a high temperature coefficient of 10^+ which may be similar to heat denaturation but occurs at a much lower temperature. The third step is the flocculation of the light and heat denatured molecules to form a visible coagulum. Radiated proteins flocculate rapidly at 40°C. if near the isoelectric point and flocculation is favored by the presence of salts.

A complete study of the influence of salts on the flocculation of radiated

protein at moderate temperatures has not been made but the method used to study the light-denaturation of isoelectric salt-free egg albumin (4) has been used to investigate the effect of ultraviolet radiation on extracts of lens protein in certain dilute salt solutions. The results confirm Burge's observations and give evidence that light, heat and calcium salts are three interrelated factors concerned in the etiology of senile and industrial cataract.

METHOD. *Lens extracts.* Some preliminary experiments were made with dialyzed α-crystallin but, in order to radiate the lens protein in its natural condition, extracts of ox lenses were made in salt solutions at 4°C. The lenses were cut in quarters and allowed to stand in the salt solution 4 hours and the solutions, which had a barely perceptible opalescence, were then filtered off, kept at 4°C. and used within 2 days. The concentration of protein was determined by boiling at the isoelectric point, filtering and weighing. If 12.5 cc. were used per lens the solutions were approximately 1 per cent protein solutions. If 15 cc. were used per lens they were 0.85 per cent solutions. The extracts had a pH of 7.0–7.2. The salt solutions used were as follows: 0.9 per cent NaCl, Ringer solution containing 0.7 per cent NaCl, Ringer solution with $CaCl_2$ added to make the $CaCl_2$ 0.05, 0.1 and 0.2 per cent, 0.45 per cent NaCl + 0.5 per cent $CaCl_2$, 0.05, 0.1, 0.2 and 0.5 per cent $CaCl_2$, 1 per cent KCl and 0.5 per cent KCl with $CaCl_2$ added to make the $CaCl_2$ concentration 0.05, 0.1, 0.2 and 0.5 per cent. All the extracts would, of course, contain some potassium from the lens itself.

Radiation. The extracts were radiated in shallow open dishes on ice, at 9 inches from a Hanovia mercury arc, for 45 minutes, which was equivalent to a dose of 100 ZnS units (1–2 ZnS units = a minimal erythema dose).

Tyndallmeter. The opalescence of the solutions was determined by means of a Tyndallmeter described in detail in a previous publication (4). The intensity of the Tyndall beam in apparent foot-candles, as read by a Macbeth Illuminometer, gives a quantitative measure of the concentration of aggregated protein when the particles are of a size to settle out from the solution. This occurs only near the isoelectric point. At a pH somewhat removed from the isoelectric point the intensity of the beam is not a strictly quantitative measure of the concentration of the aggregated protein as the intensity of the beam depends both on the concentration and the size of the particles. The readings can be taken, however, as a measure of the opalescence of the solutions, although they cannot be interpreted in grams of aggregated protein except at the isoelectric point. Tyndallmeter readings greater than 3 apparent foot-candles become inaccurate owing to the absorption of light by these dense solutions.

The Tyndall beam intensity of all the extracts was measured before

radiation or heating at pH 4.8 to 7.8, also after ultraviolet radiation (100 ZnS units) without heating, after heating to 40°C. for 2 hours without radiation, and after heating to 40°C. for 2 hours following radiation.

The pH of the solutions was determined colorimetrically.

RESULTS. *Effect of pH on opacity of solutions.* One per cent extracts of lens protein in 0.9 per cent NaCl, 1 per cent KCl, and 0.7 per cent Ringer, showed only a trace of opalescence at pH 6.4 to 7.8. The opacity began to increase at pH 6.0 and the solutions all became very dense at pH 4.8 to 5.0, which is the isoelectric point of the pseudo-globulin α-crystallin.

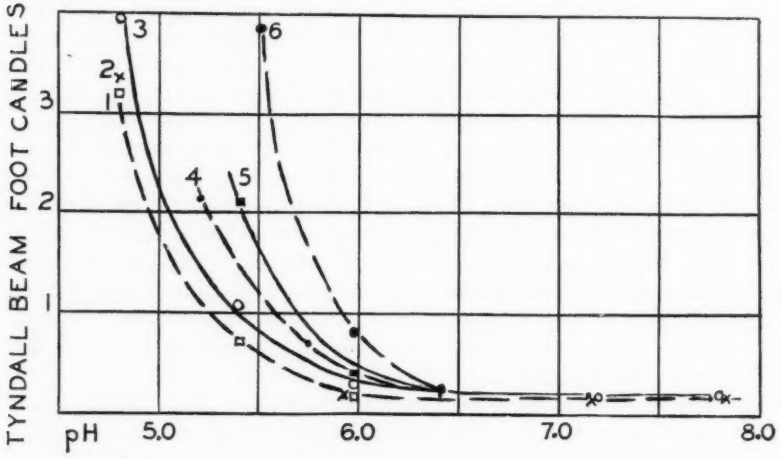


Fig. 1. Effect of pH on opacity of solutions, as measured by the intensity of the Tyndall beam. Abscissae: pH of solutions. Ordinates: intensity of Tyndall beam in apparent foot-candles. *1*, Ringer; *2*, 1 per cent KCl; *3*, 0.9 per cent NaCl; *4*, 0.5 per cent KCl + 0.05 per cent $CaCl_2$; *5*, Ringer with $CaCl_2$ added to give concentration of 0.05 per cent; *6*, 0.05 per cent $CaCl_2$.

Extracts in $CaCl_2$ (0.05, 0.1 and 0.2 per cent) showed increasing opalescence at pH 6.4 and were very dense at pH 5.4, the 0.05 per cent solution being the most dense at all pH values. A 0.5 per cent $CaCl_2$ extract however showed only a slight increase in opalescence at 4.8. This indicates a wider pH range of opalescence in the calcium proteinate extracted by these solutions and the results found with a 0.05 per cent solution are not due entirely to the low concentration of salt as a similar effect is noted when enough calcium chloride is added to 0.7 per cent Ringer solution or to 0.5 per cent potassium chloride to make the $CaCl_2$ concentration equal to 0.05 per cent. When calcium chloride is added to Ringer solution or to 0.5 per cent KCl, to give 0.1 per cent and 0.2 per cent $CaCl_2$, the opal-

escence is the same as in Ringer solution without additional $CaCl_2$ at all pH values.

Some of these results are shown graphically in figure 1 and it is evident from these curves that, although the extent of the zone of opalescence near the isoelectric point varies with the concentration and composition of the salt solution used to extract the lens protein, none of the extracts show more than a trace of opalescence at the pH of the lens, or indeed anywhere between pH 6.4 and 7.8.

Effect of moderate heat alone. All the extracts of lens proteins used were immersed in a water bath at 40°C. for 2 hours. This heating did not increase the opalescence of any extract from pH 6.0 to 7.8. At pH 5.4 there was no effect in 0.9 per cent NaCl or 1 per cent KCl and only a slight effect in Ringer solution or in Ringer containing 0.1 and 0.2 per cent $CaCl_2$, or in 0.5 per cent KCl with $CaCl_2$ (0.05–0.5 per cent) added. There was, however, a marked increase in opalescence on heating to 40°C. in $CaCl_2$ extracts (0.05, 0.1 and 0.2 per cent) and in Ringer containing 0.05 $CaCl_2$, at pH 5.4.

Effect of ultraviolet radiation alone. At pH 7.8 there was no increase in opalescence in any of the extracts except in the 0.2 and 0.5 per cent $CaCl_2$ extracts and in the 0.5 per cent KCl containing 0.2 and 0.5 per cent $CaCl_2$.

At pH 7.0 to 7.2 there was no increase in opalescence except in the $CaCl_2$ extracts and in the 0.5 per cent KCl containing $CaCl_2$. There was, however, quite a marked effect in the 0.2 and 0.5 per cent $CaCl_2$ extracts and in the 0.5 per cent KCl containing 0.2 and 0.5 per cent $CaCl_2$.

At pH 6.4 there was a definite increase in opacity in all the $CaCl_2$ extracts and in the 0.5 per cent KCl containing $CaCl_2$ and some effect in Ringer solution containing 0.2 and 0.5 per cent $CaCl_2$.

At pH 6.0 there was no increase in opalescence in Ringer, 0.9 per cent NaCl or 1.0 per cent KCl but a marked increase in all the $CaCl_2$ extracts and in the Ringer solution and 0.5 per cent KCl with $CaCl_2$ added.

At pH 5.4 all extracts showed a very definite increase in opacity. In some of the $CaCl_2$ extracts the flocculation was too dense to measure by means of a Tyndallmeter. The Ringer solution containing 0.05 per cent $CaCl_2$ was the least affected.

It is evident, therefore, that intense ultraviolet radiation alone produces no increase in opacity in extracts of lens proteins radiated at 4°C. and pH 7.2 except in the extracts containing $CaCl_2$ (0.1–0.5 per cent). Extracts in 0.5 per cent KCl containing $CaCl_2$ gave results similar to extracts in $CaCl_2$ alone but the presence of sodium chloride seemed to diminish the efficiency of calcium chloride in precipitating radiated lens protein as there was no increase in opacity in Ringer solution containing $CaCl_2$ up to 0.5 per cent. All of the solutions, however, were denatured by the

radiation as evidenced by the burnt odor and yellow color characteristic of light-denatured proteins.

Effect of ultraviolet radiation followed by moderate heat (40°C. for 2 hours). In isoelectric salt-free egg albumin it was found that, though they were clear after radiation at 4°C., flocculation developed rapidly in radiated solutions when they were heated to 40°C. for 2 hours (4).

When the radiated extracts of lens protein were heated to 40°C. many of the extracts which had shown no increase in opalescence on radiation

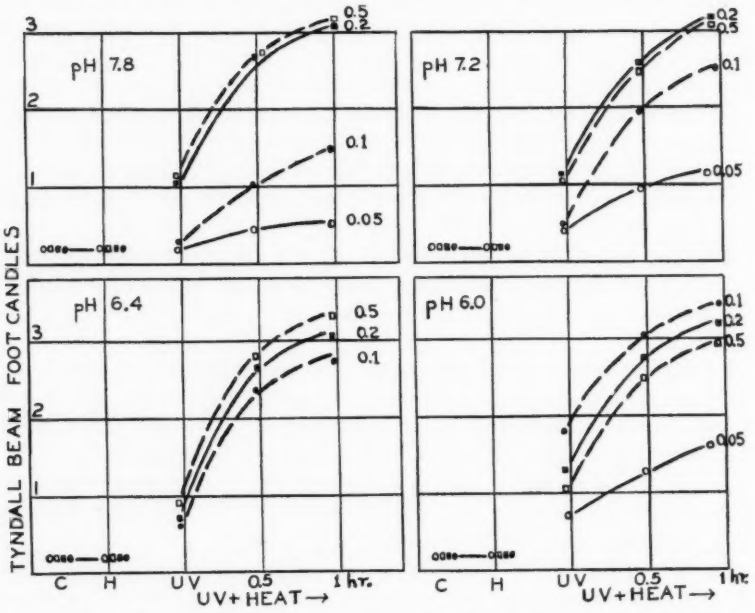


Fig. 2. Opacity of extracts of lens proteins in $CaCl_2$ solutions (0.05, 0.1, 0.2 and 0.5 per cent) at pH 6.0, 6.4, 7.2 and 7.8. Abscissae: Condition of solutions: Control, *C*, = untreated extract; heat, *H*, = extract after heating to 40°C. for 2 hours, *U.V.* = after radiation only, *U.V.* + *heat* = opacity after heating radiated extracts ½ and 1 hour. Ordinates: intensity of Tyndall beam in apparent foot-candles.

alone showed a marked increase after ½ to 1 hour at 40°C. and the opacity was greatly increased in the extracts that showed some precipitation with radiation alone.

At pH 7.8 there was no effect in 0.9 per cent NaCl, 1.0 per cent KCl, or Ringer extracts but a marked effect in all the $CaCl_2$ solutions (0.5 = 0.2>0.1>0.05 per cent), in the 0.5 per cent KCl solutions to which $CaCl_2$ had been added (0.5>0.2>0.1>0.05 per cent) and in Ringer solution containing 0.2 to 0.5 per cent $CaCl_2$.

At pH 7.0–7.2 the same was true except that the opalescence was more marked in all the $CaCl_2$ solutions (0.5 = 0.2>0.1>0.05) and in the 0.5 per cent KCl solution with $CaCl_2$ added. There was a slight increase in the Ringer solution, a marked effect in the Ringer solutions containing 0.2–0.5 per cent $CaCl_2$ and some effect in Ringer containing 0.05–0.1 per cent $CaCl_2$.

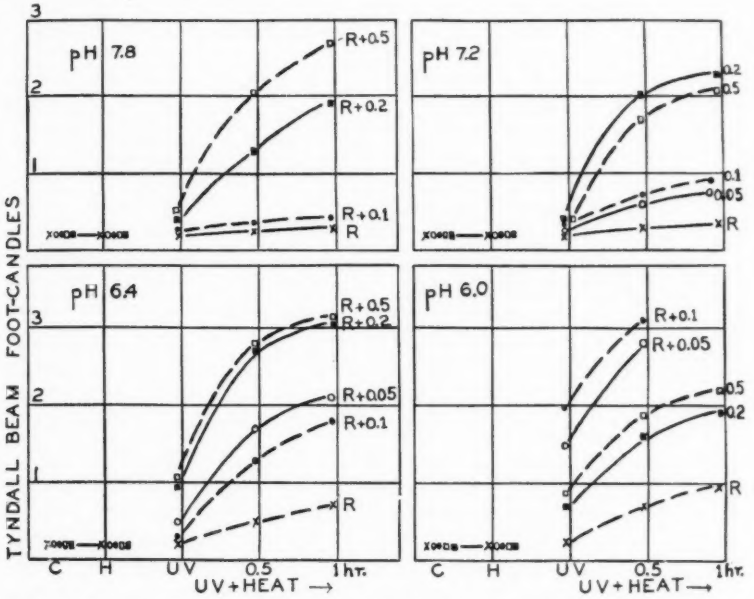


Fig. 3. Opacity of extracts of lens proteins in 0.9 per cent NaCl and Ringer solution with and without additional $CaCl_2$ (*R* = Ringer or NaCl, *R + 0.05*, *R + 0.1*, and *R + 0.2* equal Ringer with additional $CaCl_2$ to give 0.05, 0.1 and 0.2 per cent $CaCl_2$; *R + 0.5* equals 0.45 per cent NaCl + 0.5 per cent $CaCl_2$). Results are given for pH 6.0, 6.4, 7.0 and 7.8. Abscissae: Condition of solutions. *C.* = untreated extract; *H.* = extract after heating to 40°C. for 2 hours; *U.V.* = after radiation only; *U.V. + heat* = opacity after heating radiated extracts ½ and 1 hour. Ordinates: intensity of Tyndall beam in apparent foot-candles.

At pH 6.4 the opalescence was more marked in all the extracts showing an increase at pH 7.0.

At pH 6.0 and 5.4 there was increased opacity on heating after radiation even in 0.9 per cent NaCl and 1.0 per cent KCl and very dense precipitation in all other extracts.

In order to show the degree of opacity as measured by the intensity of the Tyndall beam some of the results are given in figures 2, 3 and 4. Al-

though moderate heating alone does not precipitate lens protein, and although ultraviolet radiation alone does not precipitate lens protein radiated at 4°C. at pH 7.2, except for a slight effect in the presence of dilute calcium salts, ultraviolet radiation denatures lens proteins so that when radiation is followed, or accompanied, by moderate heating (40°C. for 2 hours) there is a marked precipitation in all extracts containing dilute $CaCl_2$ (0.5>0.2>0.1>0.05 per cent). Below pH 6.4 some opacity de-

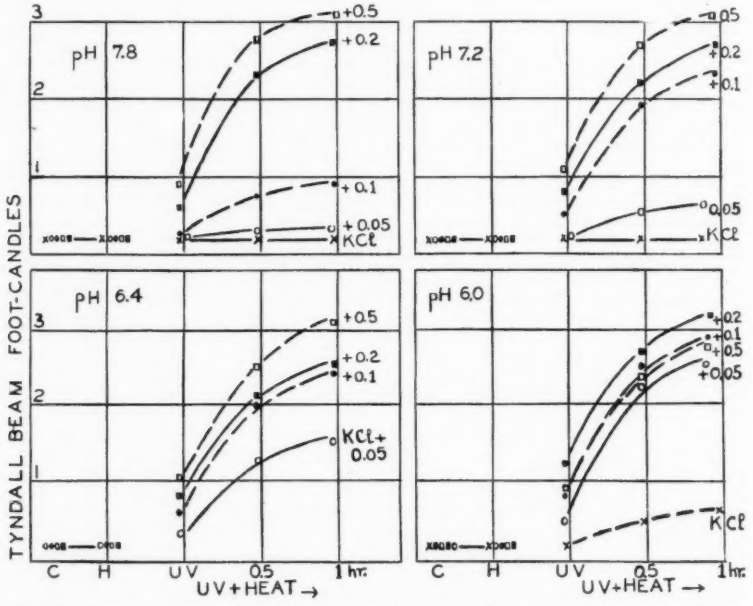


Fig. 4. Opacity of extracts of 1 per cent KCl and 0.5 per cent KCl with 0.05, 0.1, 0.2 and 0.5 per cent $CaCl_2$ added. Results are given for pH 6.0, 6.4, 7.0 and 7.8. Abscissae: condition of solutions: *C.* = untreated extract; *H.* = extract after heating to 40°C. for 2 hours; *U.V.* = after radiation only; *U.V. and heat* = opacity after heating radiated extracts ½ and 1 hour. Ordinates: intensity of Tyndall beam in apparent foot-candles.

velops in extracts containing no calcium on moderate heating after radiation.

From the standpoint of the relation of radiation to the development of cataract the results at pH 7.0 to 7.5 are the significant ones. As the lens in vivo is normally at body temperature, radiation sufficient to denature lens protein should always be followed by opalescence if calcium salts are present even in such a low concentration as 0.05 per cent. Although the effect of calcium is inhibited to some extent by the presence of sodium it is not diminished at all by the presence of potassium.

Temperature coefficient of light denaturation and coagulation in the presence of calcium. As the Tyndall beam reading is not proportional to the grams of aggregated protein except near the isoelectric point the following results obtained with 0.1 to 0.5 per cent calcium chloride extracts of lens protein at pH 7.2 are only approximate.

The temperature coefficient of the first step in the light coagulation process is only slightly greater than one. Solutions radiated at 5°C. were compared with those radiated at 15°C. after both had been heated to 40°C. for two hours. The average ratio of the opalescence $\frac{15^\circ}{5^\circ} = 1.13$. It is probable that even a temperature as low as 15°C. produces some heat change in radiated proteins in the presence of calcium so that the first step in the coagulation process is not entirely separated from the second step in this instance.

The temperature coefficient of the second step, the heat change in radiated proteins, which was found to be 10^+ in salt-free isoelectric egg albumin (4), was determined approximately by taking the ratio of the time required for a radiated $CaCl_2$ extract of lens protein to reach a certain degree of opalescence at 40°C. to that required at 30°C. The average ratio was found to be 11.4. Although these results are by no means satisfactory they show that while the presence of salts modifies the effect of radiation on proteins the effect is essentially similar to that reported for isoelectric salt-free egg albumin. The denaturation of the protein molecule by radiation has a temperature coefficient of approximately 1 and the subsequent heat change in the light-denatured molecule has a temperature coefficient approximately equal to 10.

Discussion. Duke-Elder (5) states that the primary cause of cataract is probably associated with the direct action of radiant energy on the lens increasing the lability of its colloid system and deranging the autoxidation system upon which its metabolism depends. Ultraviolet radiation may produce three changes in the eye which favor the production of cataract. One is an increase in permeability of the capsule which would favor an abnormal salt content. The second is a denaturation of the lens proteins and the third is a diminution of the glutathione content (6) which would lead to a less active metabolism. As the processes of physiological repair are slow in the lens the effect of small amounts of radiation on the denaturation of the proteins may be almost additive especially if the metabolism of the lens is decreased either by old age or by a lowered glutathione content. The effect of exposure to ultraviolet radiation on the proteins, even if additive, would not result in cataract unless calcium salts, or other substances with a similar action, are present in the lens. Adams (3) found an increase in the calcium in the blood of patients with senile cataract but as she was unable to produce coagulation of lens pro-

tein by radiation in the presence of dilute calcium salts she did not consider it significant. Her failure to produce precipitation was due to the fact that she radiated the proteins at 15°C. As the lens is normally at body temperature, and may certainly be heated to 40°C. on exposure to large sources of radiant heat, the lens protein would be precipitated in vivo by ultraviolet radiation in the presence of concentrations of calcium as low as 0.05 per cent.

Senile cataract. The radiation used throughout these experiments was the unscreened radiation from the mercury arc. As the cornea absorbs $\lambda < 295$ mμ only wavelengths longer than this will reach the lens. Burge (1) (2) however has shown that the wavelength 302 mμ, which reaches the lens and is present in sunlight, is able to coagulate lens protein in the presence of calcium. Obviously the denaturation of lens protein must occur very slowly under ordinary conditions but there must always be a certain amount of denatured protein present especially if the eye is exposed to strong sunlight. The amount of denatured protein present probably increases with age owing to a lowered metabolism in the lens. Therefore, in old age, especially in regions where strong ultraviolet radiation is present in the sunlight, there may be a relatively large amount of denatured protein in the lens. If, under these conditions, the blood calcium is increased above normal, the salt content of the lens may rise and the resulting precipitation of the denatured protein would cause senile cataract. The appearance of opacity at the periphery in senile cataract is explained by Duke-Elder (5) by the fact that this is the point where most of the fluid interchange of the lens occurs.

Industrial cataract. It has been generally agreed that heat, not ultraviolet radiation, is the cause of industrial cataracts, the heat being assumed to act indirectly by disturbing the nutrition of the lens. It is certain from these experiments that heat alone will not coagulate lens proteins at pH 7.2 even in the presence of calcium salts. However, the results reported here show that the coagulation of light-denatured proteins is greatly accelerated (temperature coefficient approximately 11) by heat. Therefore if the lens proteins are denatured by exposure to sunlight, or other radiations containing wavelengths 300–310 mμ, and if small amounts of calcium are present in the lens, exposure to molten glass and metals, by heating the lens above the normal body temperature, will greatly accelerate the precipitation of the denatured proteins. The occurrence of opacity at the posterior pole in industrial cataracts would therefore probably be due to the concentration of radiant energy at this point and the consequently higher temperature, when the eye is exposed to large sources of radiant heat.

CONCLUSIONS

Extracts of lens protein were made in solutions of sodium, potassium and calcium chloride. They were exposed to ultraviolet radiation at pH 5.4 to 7.8 and the development of opacity was followed by the intensity of the Tyndall beam from the opalescent solutions. At pH 7.2 moderate heat alone produced no opacity and ultraviolet radiation at 4°C., although denaturing the protein produced no opacity except to a slight degree in the presence of calcium. If, however, the radiated extracts were subsequently heated to 40°C. for 2 hours the opacity became very marked in all the extracts containing calcium, the effect being somewhat diminished by the presence of sodium but not by the presence of potassium.

The initial process of light denaturation may therefore occur in the lens without any visible opacity and it is probable that there is always some denatured protein present as the result of exposure to sunlight. This denatured protein does not precipitate in the presence of potassium but a calcium concentration as low as 0.05 per cent in the lens is sufficient to precipitate it at body temperature. An accumulation of denatured protein in the lens in old age combined with a higher blood calcium may therefore be responsible for senile cataract.

The second step in the light coagulation of proteins is very much accelerated by heat and occurs rapidly at 40°C. It is probable therefore that the higher incidence of cataract in workers exposed to molten glass and metals is due to an increased rate of precipitation of light-denatured protein when the lens is heated above body temperature by exposure to large sources of radiant heat, and when low concentrations of calcium or other substances producing a similar effect, are present.

REFERENCES

(1) Burge, W. E. Arch. Ophth. **38:** 435, 1909.
Arch. Ophth. **44:** 498, 1915.
Arch. Ophth. **47:** 12, 1918.
(2) Burge, W. E. This Journal **39:** 335, 1915.
(3) Adams, D. R. Biochem. J. **232:** 902, 1929.
(4) Clark, J. H. J. Gen. Physiol. (in press).
(5) Duke-Elder, W. S. Lancet **2:** 1188, 1250, 1926.
(6) Adams, D. R. Proc. Roy. Soc. B. **98:** 244, 1925.

LYMPH SUGAR

J. W. HEIM, R. S. THOMSON AND F. C. BARTTER

From the Department of Physiology, Harvard School of Public Health, Boston, Mass.

Received for publication June 15, 1935

The amount of sugar in lymph has occupied the attention of investigators from the middle of the last century to the present time. A number of workers have reported large differences between the concentrations of sugar in the lymph and blood, and although it has been rather conclusively shown that such a condition does not occur, the existence of a discrepancy between the lymph and blood sugar levels appears to be accepted by many.

A comparison of the conflicting results of previous investigators (1) is difficult to evaluate. The conditions of experimentation and of sampling, and the methods of analysis and methods of expressing the values are so varied that a common basis for comparison is not possible. The most logical method of comparing the sugar concentrations in lymph and blood are included in the following rules, most of which have been emphasized by past workers but have been repeatedly disregarded by subsequent investigators. *1.* The sugar in the lymph is in equilibrium with that in blood *plasma*. Whole blood should never be used inasmuch as it contains less sugar per unit volume than plasma due to the unequal distribution of this substance between the corpuscles and plasma. *2.* The sugar values should be expressed in terms of the water content of the fluids. *3.* Blood and lymph samples can never be simultaneously taken. It takes time for lymph to flow from its site of formation into the cannula. *4.* The lymph and blood should be taken from the same tissue area. This is especially important when dealing with thoracic-duct lymph. This lymph is a mixture which has its principal origin in the liver and intestines but also receives contributions from the limbs and other organs. It is obvious that in this case it is rather difficult to get a representative blood sample for comparison. The hepatic vein is probably the best source. *5.* It has been found by most workers that the sugar content of lymph agrees more favorably with that of arterial rather than venous plasma. *6.* The effect of anesthesia is an important one. Many have a marked influence upon the blood sugar level and the question arises as to their effect on capillary permeability and lymph filtration. Wherever possible, a general anesthetic should be avoided and the lymph vessels exposed under local anesthesia. *7.* The true or fermentable sugar of lymph and plasma should

be determined. In view of the fact that none of the older workers have satisfactorily considered all of these conditions, we have in re-investigating the subject of lymph sugar attempted to adhere to these rules.

EXPERIMENTAL. 1. *Foot lymph.* This lymph source was chosen for two reasons. The lymphatic in the ankle can be exposed and cannulated under local anesthesia with a minimum of operative manipulation. With the animal walking quietly about the laboratory, a constant flow of lymph is assured (White, Field and Drinker, 2). Blood was withdrawn by means of a syringe from the femoral artery which had also been exposed under local anesthesia. The blood sample was immediately centrifuged and the

TABLE 1

The reducing substances of arterial blood plasma and foot lymph of dogs

DOG NO.	PLASMA	LYMPH
	Normal dogs	
	mgm./100 cc.	*mgm./100 cc.*
1	121	127
2	115	107
3	104	103
4	116	115
5	112	118
6	100	106
7	107	114
8	100	100
9	133	148
10	104	112
Average.....	111	115
	Diabetic dogs	
11	537	589
12	196	193

sugar analysis begun on the plasma and lymph samples at once. The analysis was done by means of the Folin (3) micro ferricyanide method.

Table 1 gives the values of analyses of foot lymph and arterial plasma of 10 normal unanesthetized dogs. These values are based on the water contents of the fluids. In some cases the plasma sugar is higher than the lymph value, and in others the reverse is the case. The average reveals a slightly higher lymph value for the animals investigated. The difference of 4 mgm. per cent is, however, almost within the limits of experimental error.

Inasmuch as one can obtain only a very small amount of lymph from ankle vessles, it was difficult to determine the true sugar content of this

fluid. It was therefore decided to investigate the non-fermentable reducing substances in cervical lymph which can be obtained in relatively large amounts. In this case, the operative technique required the use of a general anesthesia (nembutal). The analytical procedure was as follows. Washed yeast was prepared according to the method of Trimble and Carey (4). The fermentable sugar was removed by means of this preparation from diluted plasma and lymph samples, from which the proteins were precipitated by means of tungstic acid and removed by centrifuging. The reducing values of this fluid were determined by means of the Folin micro method.

Table 2 shows the results of this experiment. If we base the values on the water content, the average values become 5.9 mgm. per cent for plasma and 6.0 mgm. per cent for lymph, that is, they are practically

TABLE 2

The non-fermentable reducing substances of blood plasma and cervical lymph

DOG NO.	PLASMA	LYMPH
	mgm./100 cc.	*mgm./100 cc.*
13	5.9	5.5
14	6.3	6.4
15	5.4	7.1
16	10.5	7.1
17	5.0	5.8
18	5.8	7.4
19	4.6	5.7
20	4.0	4.0
21	4.4	4.7
22	3.3	4.0
Average.....	5.5	5.8

identical. If we assume that foot lymph possesses the same amount of non-fermentable reducing substances as cervical lymph, by subtracting this value (6 mgm. per cent) from the averages of table 1 we get 105 mgm. per cent for plasma and 109 mgm. per cent for lymph. The sugar values of two diabetic dogs are also given in table 1. Inasmuch as only two animals were studied, one cannot form any definite conclusions from these figures. Dog 11 shows a wide divergence between lymph and plasma values, although the per cent difference does not exceed that found in many normal dogs. The difference in dog 12 is small and in the opposite direction. This is in accord with the findings among normal animals. Unfortunately, due to the fact that only small amounts of lymph could be obtained, no fermentation experiments could be done. It may be possible that the large difference between the plasma and lymph values of dog 11

is due to a larger amount of non-fermentable reducing substances in the lymph.

2. *Intestinal lymph.* We have attempted to duplicate the experiments of Fischer and Winter (1) on the sugar in the intestinal lymph of cats in view of reëxamining the large discrepancies which they found between intestinal lymph and blood sugar levels. The animal was given a general anesthetic, the abdomen opened, and the intestines withdrawn as far as necessary. The lymphatic trunk which collects the lymph from the

TABLE 3

The reducing substances of intestinal lymph

Cat 1. Male, 3.4 kgm. Nembutal anesthesia

TIME	LYMPH	PLASMA
	mgm./100 cc.	*mgm./100 cc.*
11:10	247	
11:15	236	
11:25		222 (arterial)
11:38		219 (venous)
11:50	219	

TABLE 4

The effect on lymph sugar of passage through a node

Dog 23. Male, 22.2 kgm. Nembutal anesthesia

TIME	TOTAL REDUCING SUBSTANCES	
	Afferent lymph	Efferent lymph
	mgm./100 cc.	*mgm./100 cc.*
12:04	124	120
12:20	116	114
12:43	108	105
1:14	105	102
2:00	122	125
2:30	124	126
Average.....	116	115

various intestinal nodes was cannulated and the lymph sampled and analyzed for sugar as described in the last section. Although a variety of anesthetics were used (nembutal, dial, chloralose) it was found impossible to perform the operation without evoking a considerable increase in the blood sugar. In none of the experiments did we ever obtain differences of the magnitude reported by Fischer and Winter. Table 3 shows results of a typical experiment. In attempting to investigate the observation that sugar is removed from lymph during its passage through a node,

we found it impossible to secure sufficient lymph from afferent and efferent intestinal node vessles for analysis. We did, however, succeed in obtaining an abundant supply by cannulating the vessels entering and leaving the popliteal node of the legs of dogs. Nembutal anesthesia was used and cannulas were placed in the efferent and afferent vessels. As indicated in table 4, there is no appreciable drop in the lymph sugar on passage through the node.

3. *Lymph sugar following sugar administration.* Dogs weighing in excess of 20 kgm. were anesthetized with nembutal given intraperitoneally. The cervical lymphatic trunks were exposed and cannulated. Blood was

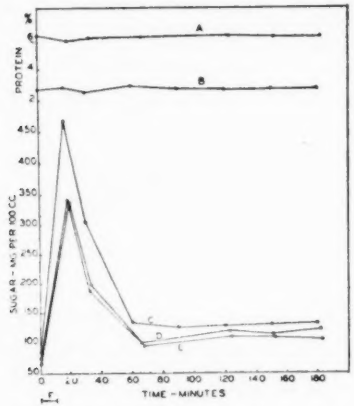


Fig. 1

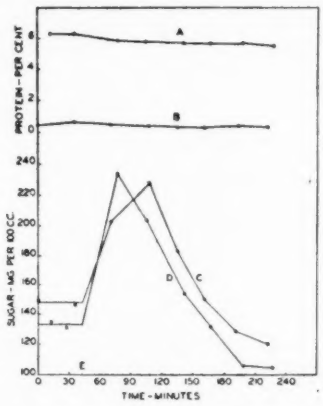


Fig. 2

Fig. 1. Dog 24. Male, weight 21 kgm. Nembutal anesthesia. A, plasma protein; *B*, lymph protein; *C*, lymph sugar; *D*, arterial plasma sugar; *E*, venous plasma sugar; *F*, 1.0 gram dextrose per kilogram administered intravenously.

Fig. 2. Dog 25. Male, weight 25 kgm. Local anesthesia. *A*, plasma protein; *B*, lymph protein; *C*, lymph sugar; *D*, plasma sugar; *E*, 2.0 grams dextrose per kilogram administered by stomach tube.

withdrawn from the cervical vein and carotid artery, transferred to oxalated tubes, immediately centrifuged and the plasma sampled. The sugar was administered by means of a 50 per cent aqueous solution of Pfanstiel's C. P. anhydrous dextrose (prepared for intravenous injection). This solution was injected into a femoral vein at a uniform rate in ten minutes. The animals received 1 gram of dextrose per kilogram of body weight. Following the sugar injection, no lymphagogue action was ever noted nor was a marked change in concentration (as indicated by protein determinations) ever observed.

Figure 1 shows typical glucose tolerance curves of lymph and blood plasma. It will be noted that the lymph sugar content during the normal, hyperglycemic and hypoglycemic blood sugar phases is always greater

than the plasma level, and that at the maximum hyperglycemic stage, the lymph sugar rises to a considerably higher value than the corresponding plasma sugar level. The large discrepancy between the maximum hyperglycemic values should not be regarded as significant inasmuch as these levels are maintained in the blood and lymph for a very short time only and are easy to miss unless many samples are taken. These curves illustrate the rapidity with which the sugar enters the lymphatic system, and indicate that it is removed from lymph at approximately the same time as from the blood. The discrepancy between the curves of plasma and lymph may be attributed to two causes, a time lag in the lymph sampling and possibly to the effects of anesthesia. The first of these factors cannot be avoided. It takes time for the newly formed lymph to reach the cannula and we have no way of materially reducing this interval. Effects of anesthesia can be eliminated by obtaining foot lymph from unanesthetized dogs. In these experiments foot lymph and arterial plasma were sampled as has already been described. Normal samples were taken in duplicate and were followed by the administration of 2 grams of dextrose per kilogram of body weight by stomach tube. Figure 2 shows the result of such an experiment. The first observation of importance is the fact that the normal lymph values are 15 mgm. per cent higher than the corresponding plasma values. This occurrence is occasionally found and as yet no adequate explanation can be offered to account for it. The sugar enters the lymph stream rapidly as indicated by the almost parallel rise in the lymph and plasma curves. As previously discussed, it is debatable whether the maximum values found here are really the true maximum levels. At all events, the highest lymph value is less than the maximum plasma level. The decline in lymph sugar follows closely that of plasma, the slight discrepancy being probably due to a time lag. On the whole, these curves do not essentially differ from those for cervical lymph during anesthesia.

SUMMARY

1. A set of conditions which must be observed in order to establish a true basis for a comparison of the blood and lymph sugar levels has been formulated.

2. When these conditions are satisfied, the sugar contents of lymph and plasma were found to be nearly the same.

3. The glucose tolerance curves for simultaneously collected lymph and plasma specimens of anesthetized and unanesthetized dogs have been determined. The curves for lymph very closely resemble those of plasma.

4. When sugar is administered either by intravenous injection or by stomach tube, it enters the lymphatic system very readily, and is removed from the lymph at approximately the same rate as from blood.

REFERENCES

(1) Arnold, R. M. and L. B. Mendel. J. Biol. Chem. **72:** 189, 1927.
Clark, G. A. and L. B. Winter. J. Physiol. **83:** 49P, 1935.
Cohnstein, W. Pflüger's Arch. **62:** 58, 1895.
Fay, M. and P. S. Wharton. J. Biol. Chem. **109:** 695, 1935.
Fischer, F. P. and L. B. Winter Biochem. Ztschr. **270:** 157, 1934.
Heidenhain, R. Pflüger's Arch. **49:** 209, 1891.
Hendrix, R. M. and J. E. Sweet. J. Biol. Chem. **32:** 299, 1917.
Katsura, S. Tohoku J. Exp. Med. **7:** 382, 1926.
Meyer-Bisch, R. and F. Günther. Pflüger's Arch. **209:** 81, 92, 107, 1925.
Nasse, H. 1872. (Quoted by Heidenhain, 1891.)
Osato, S. Tohoku J. Exp. Med. **2:** 489, 1921.
Rony, H. R., B. Mortimer and A. C. Ivy. J. Biol. Chem. **96:** 737, 1932.
Von Mering. Arch. f. Anat. u. Physiol. 379, 1877.
(2) White, J. C., M. E. Field and C. K. Drinker. This Journal **103:** 34, 1933.
(3) Folin, O. and A. Svedberg. J. Biol. Chem. **88:** 85, 1930.
(4) Trimble, H. C. and B. W. Carey. J. Biol. Chem. **90:** 655, 1931.

REFLEX LIBERATION OF CIRCULATING SYMPATHIN

A. C. LIU[1] AND A. ROSENBLUETH

From the Laboratories of Physiology in the Harvard Medical School

Received for publication June 17, 1935

Direct stimulation of sympathetic nerves leads to the passage into the blood of sympathin, a sympathomimetic substance whose presence may be demonstrated by reactions of adequate denervated autonomic effectors (for references see Cannon, 1933). For this passage to occur a certain degree of stimulation is necessary, as indicated by the existence of a threshold frequency of maximal stimulation below which no sympathin is demonstrable (Rosenblueth and Morison, 1934). The question arises, can sympathin be produced by reflex activation of sympathetic nerves; that is, can this reflex activation attain the degree necessary for the passage of sympathin into the blood? The present study attempts to answer this question. The issue is intimately related to the possible rôle of sympathin as a hormone in physiological conditions.

METHOD. Cats were used. The right superior cervical sympathetic ganglion was removed and time allowed for degeneration of the fibers to the nictitating membrane (n.m.) and for sensitization of this muscle to sympathin (6 to 20 days). The cats were then anesthetized with ether succeeded by urethane (1 to 1.5 grams per kgm.). The adrenal glands were removed. The vagi were usually cut. Curare, in addition to the urethane, was injected when movements of the eye confused the interpretation of contractions of the n.m.

The blood pressure was recorded from the left carotid artery by a mercury manometer. The contractions of the denervated right n.m., used as an indicator of sympathin, were recorded isotonically.

The sciatic or one of the brachial nerves was employed as the afferent. They were dissected, cut peripherally and placed on shielded buried electrodes. A Harvard inductorium furnished the stimuli applied.

RESULTS. Central stimulation for 30 to 60 seconds at tetanizing frequency of either of the afferents mentioned evokes a contraction of the n.m. The onset of this response occurs 15 to 60 seconds after the beginning of stimulation. The maximum is reached 1 to 3 minutes later. Subsidence of the contraction takes usually several more minutes (2 to 6). Figure 1 illustrates a typical instance. The record is closely similar to

[1] Fellow of the Peiping Union Medical College, China.

those obtained by direct stimulation of sympathetic nerves which leads to liberation of sympathin (cf. Rosenblueth and Cannon, 1932).

Repeated stimulations of the afferent lead to a progressive decline of the responses of the n.m. so that after 6 to 10 observations, separated by intervals of about 10 minutes, practically no contraction ensues. It is noteworthy that although the pressor reflex elicited shows also a gradual decline it is not as pronounced as that presented by the n.m., so that marked blood-pressure effects may be recorded without any significant contraction of the n.m.

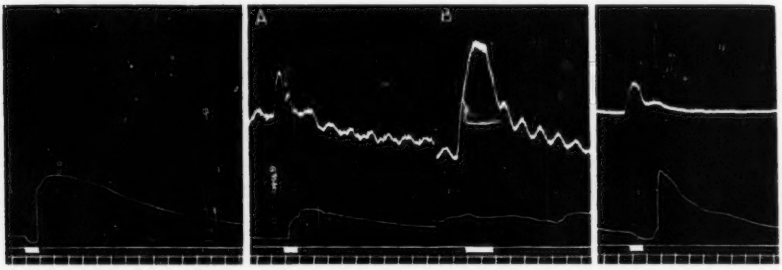


Fig. 1 Fig. 2 Fig. 3

Fig. 1. Urethane. Curare. Adrenals tied. Vagi cut. Contraction of the right nictitating membrane denervated 6 days previously on central stimulation of the left sciatic (coil distance: 6 cm.) at the signal. In this and the succeeding figures time is recorded in 30-second intervals.

Fig. 2. Urethane. Adrenals tied. Vagi cut. Hepatic nerves cut. At signals central stimulation of the right sciatic.

A. Before removal of the stellate ganglia. Coil distance: 7 cm.

B. After removal of the stellate ganglia. Coil distance: 4 cm.

Fig. 3. Urethane. Curare. Adrenals tied. Vagi cut. Hepatic nerves cut. Stellate ganglia removed. At signal central stimulation of the right sciatic; coil distance: 6 cm.

The responses of the n.m. are a function of the amount of intact sympathetic nerves present in the animal. Thus removal of the stellate ganglia, or section of the hepatic nerves, or exclusion of the caudal part of the animal by clamping the aorta at the diaphragm leads to a marked decrease in the responses or to their total disappearance. There are considerable differences from this standpoint in different animals. In some instances it suffices to remove the stellate ganglia or sever the hepatic nerves to abolish the contractions of the n.m. (fig. 2), while in other cases it is possible to record large responses after the two operations are performed (fig. 3).

Stimulation of the afferents is successful in evoking n.m. contractions only if it induces well-defined increase of sympathetic activity-rise of

blood pressure and acceleration of the heart after section of the vagi. Weak stimuli may therefore be insufficient. Strong stimuli may also fail to elicit any n.m. response if applied to a background of marked sympathetic hyperactivity. Thus in one cat with the aorta clamped at the diaphragm and the vagi cut the heart rate was 210 per minute. Stimulation of a brachial nerve increased this rate to 225, while the n.m. did not contract above its previous level. The rate of 210 before stimulation is indicative of strong sympathetic discharge. That sympathin was probably constantly passing into the blood was shown by the persistent contraction of the n.m. and protrusion of the eye. The stimulus failed then to increase significantly the concentration of this sympathin in the blood.

The responses of the n.m. are independent of the basal blood pressure. After clamping the aorta at the diaphragm a high blood pressure is the rule (above 160 mm. Hg), yet no contractions are obtainable after removal of the stellates. In other instances, of which figure 3 is an example, after removal of the stellates and section of the hepatic nerves the n.m. still responded, although the blood pressure was only 80 mm. Hg.

Discussion. A humoral factor was responsible for the contractions of the n.m. since the muscle was denervated. Adrenine was invariably excluded.

Rises of blood pressure were not responsible, for after repeated stimulations they persisted while the n.m. failed to contract. Further, clamping the aorta at the diaphragm, which resulted in a marked rise of blood pressure, did not evoke a response of the n.m. Further still, the changes of blood pressure and the contractions of the n.m. did not follow a parallel course (figs. 2 and 3). Indeed, in these and in previous observations, when sympathetic nerves have been stimulated that lead to a rise of blood pressure, an apparent *relaxation* of the n.m. occurs shortly after the beginning of stimulation, too early for a humoral effect, and correlated with the rise in blood pressure (figs. 1 and 3).

Changes in the blood referable to respiratory effects were excluded by the observations on curarized animals with uniform artificial respiration.

Some hormone other than adrenine is therefore responsible for the contractions of the n.m. recorded. That the sympathetic controls the production of this hormone is shown by the dependence of the results on the amounts of sympathetic nervous system intact in the animal and by the lack of results if sufficient sympathetic is removed. That it is not a specific secretion of a specific gland is shown by the irrelevance of what portions of the sympathetic were intact when the responses occurred—i.e., contractions ensued when the thoracic sympathetic was in and the cephalic and abdominal sympathetic out, or when the cephalic and thoracic elements were inactivated and some abdominal and somatic (hind limbs and tail) portions were functionally available.

The only known substance that may explain the results is sympathin liberated by reflex activation of the sympathetic. The appearance of sympathin in the blood has been demonstrated when sympathetic nerves are stimulated directly (see introduction), during emotional excitement (Newton, Zwemer and Cannon, 1931), during the hyperactive periods of a decorticate cat (Whitelaw and Snyder, 1934) and in physiological states which involve hyperactivity of the sympathetic such as cold and hypoglycemia (Partington, in preparation). From these reports and from the present study on reflex production of sympathin we may conclude that all conditions which involve sufficient sympathetic activity in some territory lead to the passage of sympathin into the blood and a possibility of generalized sympathomimetic effects.

Sympathin may then be significant as a hormone synergistic with adrenine and insuring widespread activation of effectors supplied by the sympathetic. It is pertinent to recall in this respect the fact emphasized by one of us (Liu, 1935) that small quantities of sympathin from a source which may have no measurable effects on a given indicator distinctly increase the responses to other modes of activation of the effector (stimulation of its nerve supply, adrenine or sympathin from other sources).

Although we conclude that sympathin may be significant as a hormone in several physiological conditions, we do not wish to overestimate its importance. In all the experiments devised to demonstrate circulating sympathin the indicators have been rendered abnormally sensitive to the hormone by previous denervation or by cocaine. If these sensitizing procedures are not employed the effects of sympathin are small or absent. Three distinct degrees of activity are recognizable when sympathin is liberated: a maximum effectiveness at the site of production, correlated with the maximum concentration; intermediate concentration and activation at neighboring cells in the source, cells either not innervated or whose nerves did not discharge; finally, minimum concentration and responses in distant effectors. The latter is probably significant only during considerable widespread sympathetic discharges.

SUMMARY

Stimulation of an afferent nerve in adrenalectomized cats under urethane anesthesia may lead to a delayed contraction of the denervated nictitating membrane (fig. 1). The response is dependent on how many sympathetic nerves are intact in the animal and available for reflex activation (figs. 2 and 3). It is concluded that the agent responsible for the contractions is sympathin liberated reflexly into the blood (p. 557), and that sympathin may therefore play a rôle as a hormone in certain physiological conditions (p. 558).

REFERENCES

CANNON, W. B. Science **78:** 43, 1933.
LIU, A. C. This Journal, **112,** 690, 1935.
NEWTON, H. F., R. L. ZWEMER AND W. B. CANNON. Ibid. **96:** 377, 1931.
ROSENBLUETH, A. AND W. B. CANNON. Ibid. **99:** 398, 1932.
ROSENBLUETH, A. AND R. S. MORISON. Ibid. **109:** 209, 1934.
WHITELAW, G. P. AND J. C. SNYDER. Ibid. **110:** 247, 1934.

REVERSIBLE LOSS OF THE ALL OR NONE RESPONSE IN COLD BLOODED HEARTS TREATED WITH EXCESS POTASSIUM

GEORGE H. ZWIKSTER AND T. E. BOYD

From the Department of Physiology and Pharmacology, Loyola University School of Medicine, Chicago, Illinois

Received for publication June 24, 1935

While studying the effects of cooling, in frog and turtle hearts treated with modified Ringer's solutions, we noted, as Martin (1904) did, that the temperature of cold standstill is relatively high in hearts exposed to an excess of potassium. At the temperature of stoppage, the hearts still gave the typical all or none response to artificial stimuli. On further cooling for several degrees, however, auricles often showed graded contractions with graded stimuli, returning to the usual all or none behavior on rewarming. Further investigation showed that, in ventricles kept at room temperature, the graded type of response could be brought on by the action of excess potassium alone; and that the effect was reversed on replacing the preparation for a time in normal Ringer's solution.

The experiments were carried out from September, 1934, to the following June. The turtles used were nearly all *Pseudemys elegans*, with a few of *Chrysemis marginata*. Bullfrogs (*R. catesbiana*) were used during May.

Our normal turtle Ringer's solution was of the composition recommended by Baetjer and McDonald (1932), containing NaCl 0.700, KCl 0.040, and $CaCl_2$ 0.025 per cent. This was modified by increasing the KCl and at the same time reducing the NaCl sufficiently to keep the total osmotic pressure constant. The effects described below required, for turtle hearts during the winter months, 4 or 5 times the normal concentration of KCl (0.16 to 0.20 per cent). In late spring this had to be increased to 0.24 per cent or more.

When a ventricle or ventricular strip is placed in the modified Ringer solution containing 0.20 per cent KCl ("5K Ringer") it usually ceases, within 1 to 2 hours, to respond to induction shocks or condenser discharges of ordinary intensity and duration. This effect of K on heart muscle has been known since the work of Ringer (1887). Such a preparation can still be made to contract, however, by constant currents of sufficient voltage and duration. We have used as electrodes, in some experiments, small silver wires coated with AgCl and hooked into the muscle; in others,

Ag-AgCl electrodes immersed in NaCl solution, from which yarn wicks led to the muscle. Using either isotonic or tension levers for recording, graded responses can be shown by the following procedures:

1. By varying the voltage, using constant duration (ordinarily, in our experiments, about 2 sec.). The threshold is high, usually 5 to 10 volts, and 80 or 90 volts may be required to bring out a maximum response (fig. 1A).

2. By varying the duration, using a constant high voltage. Figure 1B shows the responses to stimuli of 50 volts, varying in duration from 0.1 to 3.0 seconds. We have not made a systematic study of time-voltage relations, but have made chronaxie measurements on a few preparations, obtaining values of 0.100 to 0.130 second.

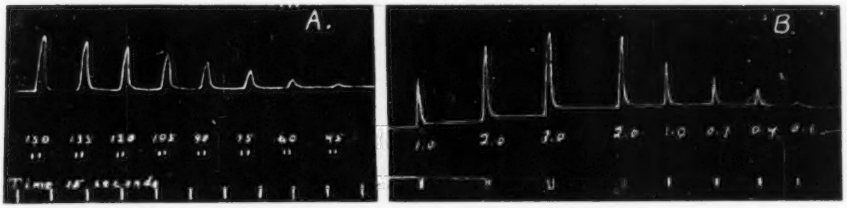


Fig. 1. A. Isotonic contractions of bullfrog ventricle, soaked for 1 hour in 5K turtle Ringer, 5/25/35. Stimulus duration about 3 seconds, voltage varied as indicated. The high voltages necessary here, and also in figure 2, were due mainly to the resistance (approximately 30,000 ohms) of the non-polarizable electrodes used.

B. Turtle ventricle, soaked for 2 hours in 4K Ringer, 4/17/35. Stimulated through metallic (Ag-AgCl) electrodes. Voltage constant at 50, duration varied and indicated in seconds.

3. By varying the position of the electrodes, keeping both voltage and duration constant. This can be most readily demonstrated on strips. The height of the recorded contraction increases as the electrodes are placed further apart, indicating, apparently, that only the tissue traversed by the current is taking part in the contraction.

Kronecker (1883) and several later workers found an apparent failure of the all or none response in dying or narcotized hearts. Kato (1926, pp. 153–160) describes experiments carried out in his laboratory by T. Ota on the narcotized toad's heart. Ota found in a few cases, in advanced narcosis, contractions varying in height with the strength of the stimulus. Kato explains these as due probably to impaired conduction, either *a*, localized blocks, excluding certain parts of the heart from the response until the stimulus was made strong enough to reach them directly, or *b*, such a degree of slowing that, with weak stimuli, the different parts of the heart were not all acting simultaneously upon the recording device.

The true criterion for all or none behavior, according to him, is "the contraction degree of each cell." Consequently he does not regard the variations found by Ota and others to be real exceptions to the principle.

Gilson (1935) has repeated some of our experiments. He agrees that the time factor of stimulation is profoundly changed, and that with increasing voltages the response spreads to include more and more of the ventricle. From direct observation of the latter effect he believes that it is due to localized blocks. We also have seen preparations in which such blocks were evident. They were, however, preparations which had been soaked for a short time, or in relatively weak concentrations of KCl. We are inclined, therefore, to believe that localized blocks exist only during an intermediate stage of the action of potassium. With the heart divided into fractions, each responding all or none, the number of possible variations in contraction height would be limited by the number of such isolated fractions which could be successively brought into activity. A ventricle soaked for 2 hours in 5K Ringer can be made to show any desired number of steps between the base line and the maximum contraction, if the stimuli are uniform in duration and properly graded as to intensity. Such behavior, it seems to us, can hardly be explained on the basis of localized blocks. It might be attributed either to *a*, conduction with a decrement, or *b*, total failure of conduction, the contractile mechanism remaining capable of activity when directly stimulated.

Kato's second hypothesis, that of delayed conduction, also seems inadequate to explain the gradations, though slowed conduction may occur in the intermediate stage referred to above. It would involve, as he points out, a considerable prolongation, in time, of the total cardiac response to a weak stimulus. Kronecker (loc. cit.) noted, in hearts in poor condition, a slow peristalsis-like contraction set up by relatively weak stimuli. We have never seen, however, in preparations showing complete gradation, a recorded mechanical response of longer duration with weak than with strong stimuli.

The activity described seems to us to be a galvanic contracture, similar to that of skeletal muscle. Mines (1908) showed that in excess KCl the frog's sartorius lost its twitch response, with retention of the contracture. Saito (1906) made similar observations on muscles treated with various narcotizing agents. Bremer (1932) finds that the chronaxie for the contracture of the sartorius is about 100 times as long as the chronaxie for its twitch. Lucas (1907) reported that the contracture of the sartorius is independent of the rate of intensity change of the stimulus. We have applied constant currents to the potassium-treated turtle ventricle, beginning with zero voltage and turning a potentiometer slowly by hand so that the maximum was reached in one minute or more. The ventricle contracts progressively, relaxing when the circuit is broken (fig. 2C). The

maximum shortening reached, however, is not so great as that obtained when the maximum voltage is applied as a rectangular shock of 3 seconds' duration. This difference is probably due to a "fatigue" developing with the prolonged passage of a one-way current. After repeated stimulation with currents of the same direction, the response to a given voltage is usually improved by reversing the polarity of the electrodes (figs. 2A, 3B).

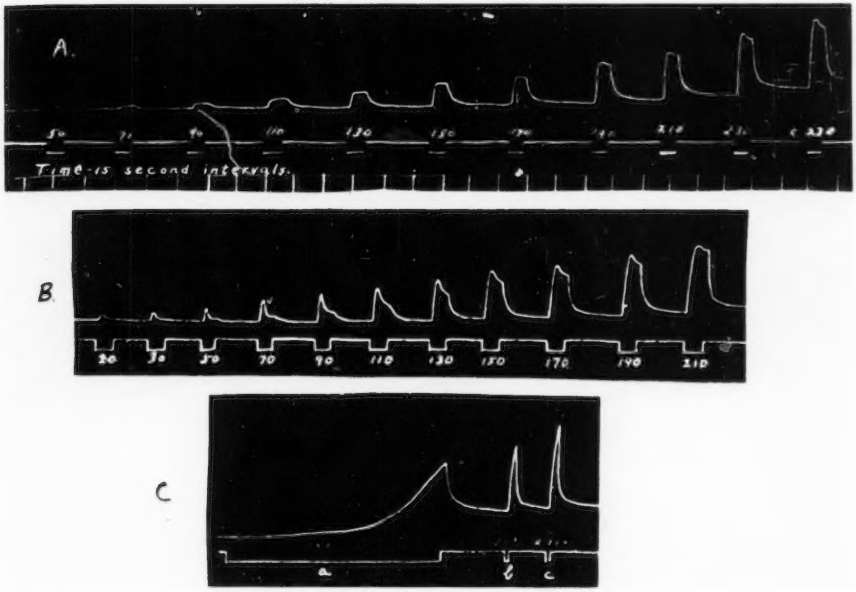


Fig. 2. A. Turtle ventricle, in 5K Ringer for 2 hours, 6/4/35. Stimulation with constant currents of 10 seconds' duration, voltage varied as indicated. Electrodes reversed at *R*.

B. Same preparation, stimulated in same manner, 30 minutes later.

C. Same preparation. At *a*, applied constant current rising gradually from 0 to 250 volts over period of 130 seconds; *b*, 250 volts for 3 seconds, polarity same as in *a*; *c*, repetition of *b* with electrodes reversed.

Relaxation usually is prompt following the break of the stimulating circuit. With a brief stimulus (2 seconds or less) the duration of the response is approximately the same as that of a normal beat. With prolonged constant currents (10 seconds or more) the response may be recorded as a simple plateau (fig. 2A). In other cases, the weakest effective voltage may elicit a "make" contraction only; intermediate, a sustained plateau on which the "make" contraction is superimposed, while with high voltages the initial peak is eliminated (fig. 2B). The galvanic con-

tracture in skeletal muscle is usually described as maintained for the duration of the current flow, but Saito (loc. cit.) shows records from the sartorius which resemble figure 2B.

In skeletal muscle, again, the galvanic contracture with weak currents is described as being localized at the cathode. With a stigmatic cathode and direct observation, this can be demonstrated on the heart. Relatively low voltages, however, evoke activity at considerable distances from the cathode. Figure 3A shows the behavior of a ventricle with one electrode and a muscle lever attached to each lateral extremity. The ventricle was fixed at the mid-line in a Gaskell clamp which was tightened just sufficiently to prevent the mechanical pull of either half from affecting the opposite lever. It will be seen that graded responses appear on the anodal as well as the cathodal side. When both electrodes are on the same side

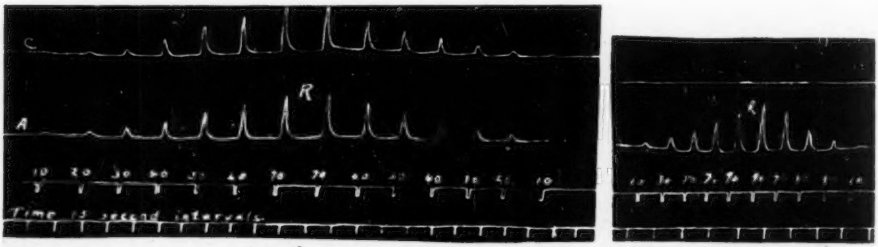


Fig. 3. A. Turtle ventricle, in 6K Ringer for 4 hours, 6/7/35. A anodal, C cathodal side, electrodes reversed at *R*. Resistance of electrodes approximately 15,000 ohms.
B. Same preparation, arrangement unchanged except that both electrodes are on same side of clamp, cathode medial. Responses on side of electrodes (second line), none on opposite side (top line). Polarity reversed at *R*. For further description see text.

of the clamp the opposite half of the ventricle fails to respond (fig. 3B); and we are convinced, having repeated the procedure with varying adjustment of the clamp, that the failure is not due to a pressure block. The wick electrodes, in this instance, made contact with the tissue over an area of approximately 10 sq. mm.

We have seen ventricles, behaving toward constant currents in the manner described, fail entirely to respond to break shocks from a Porter induction coil, even with 22.5 volts in the primary circuit and with the secondary coil at 0 distance. Frequently, however, such strong shocks will cause the heart to enter into a state of sustained contracture from which it relaxes slowly or not at all. Relaxation is occasionally incomplete after brief constant-current stimuli (fig. 1B). This is particularly true when polarizable electrodes and high voltages are used. Carlson

(1906) observed a somewhat similar after-contracture in the invertebrate heart, and Rössler (1924) in that of the frog. In our experience it is more often seen in auricles than in ventricles.

Smooth muscle is known to be present in both auricles and ventricle of the turtle (Shaner, 1923). We have not been able to find a definite statement as to its presence in the frog's ventricle. It must therefore be considered possible that the graded effects are due to the activity of

TABLE 1

Potassium gain or loss of turtle ventricles soaked in normal or modified Ringer solutions

Normal turtle Ringer, NaCl 0.70, KCl 0.04, $CaCl_2$ 0.025 per cent. All left at room temperature, 18 to 22°C.

SOLUTION	TIME OF SOAKING	WET WEIGHT OF VENTRICLE	K GAIN OR LOSS		CONDITION OF VENTRICLE ON REMOVAL
			Total	Per gm. of ventricle	
	hours	*gm.*	*mgm.*	*mgm.*	
Normal Ringer (0.04 per cent KCl)	18	1.34	−0.48	−0.36	All or none
	8	1.40	0.0	0.0	All or none
	24	1.59	0.0	0.0	All or none
3K Ringer (0.120 per cent KCl)	1.75	1.90	0.0	0.0	Gradations (partial)
	7	1.5	+1.58	+1.05	Gradations
	3.5	1.23	+0.52	+0.42	Gradations
4K Ringer (0.16 per cent KCl)	3.0	1.20	+1.47	+1.23	Gradations
	2.0	1.39	+1.58	+1.14	Gradations
	1.0	1.05	+1.20	+1.14	Gradations
	1.0	1.30	+1.64	+1.26	Gradations
	1.0	1.16	+1.20	+1.04	Gradations
	1.5	1.20	+0.99	+0.83	Gradations
5K Ringer (0.20 per cent KCl)	3.0	1.40	+1.37	+0.98	Gradations
	2.0	1.18	+0.95	+0.81	Gradations
	1.0	1.20	+1.82	+1.52	Gradations
6K Ringer (0.24 per cent KCl)	2.5	1.54	+2.20	+1.43	Gradations

smooth muscle. This seems to us unlikely, however, because *a*, the ventricular responses seem to be too powerful to be accounted for by the small quantity of smooth muscle present, and *b*, smooth muscle could hardly be expected to imitate so closely the time course of a normal beat.

It is well known that frog hearts regain normal excitability and spontaneous activity in Ringer's solution, after exposure to even higher concentrations of K than any used by us (Burridge, 1932). Our preparations

have uniformly recovered normal behavior in Ringer. The graded responses are not, therefore, attributable to a dying condition of the tissue.

Ventricles show graded responses after soaking in a solution containing 0.2 per cent KCl with a proportionate increase of $CaCl_2$. The threshold, however, is relatively low, and the responses appear to be fractional. Higher concentrations of K bring on perfectly graded responses, even when the K/Ca ratio is kept constant.

Horton (1932) studied the condition of inexcitability produced by soaking the excised frog's sartorius in potassium-Ringer. He found that it was not necessary for the muscle to take up K from the solution in order to become inexcitable, but only that a state of approximate diffusion equilibrium should exist. Retention of excitability seemed to require a constant loss of K.

We have made some studies on the potassium balance with turtle ventricles. They were washed free of blood in Ringer's solution immediately after excision, weighed, and placed in either 15 or 25 cc. of the modified Ringer. After a varying time at room temperature they were removed and the character of the response to stimulation noted. The final K content of the solution was determined by the method of Breh and Gaebler (1930) and compared with the original. The figures are in table 1. It will be seen that, of 14 ventricles which showed a failure of the all or none response, 13 gained K from the solution. Ventricles left in normal Ringer seemed to be in approximate diffusion equilibrium, though the number of such preparations is perhaps too small to permit the making of a general statement.

SUMMARY

1. The ventricle of the turtle or bullfrog heart, soaked for 1 to 2 hours in modified Ringer solution containing 0.2 to 0.4 per cent KCl, shows perfectly graded mechanical responses to graded stimuli.

2. Stimuli of brief duration are relatively or entirely ineffective. The chronaxie is of the order of 0.125 second at 20°. Gradations of response may be obtained either by using constant duration and varying voltage, or by using constant voltage and varying duration.

3. It is concluded that this type of activity is probably a contracture, fundamentally similar to the galvanic contracture of skeletal muscle.

4. Ventricles behaving in the manner described are found to have taken up K from the modified Ringer solution.

5. Normal response to stimulation is restored when the preparations are soaked for a time in normal Ringer solution.

REFERENCES

BAETJER, A. M. AND C. H. MCDONALD. This Journal **99:** 666, 1932.
BREH, F. AND O. H. GAEBLER. J. Biol. Chem. **87:** 81, 1930.

BREMER, F. J. Physiol. **76:** 65, 1932.
BURRIDGE, W. Excitability, a cardiac study. Oxford, 1932.
CARLSON, A. J. This Journal **16:** 85, 1906.
GILSON, A. S. Personal communication, 1935.
KATO, G. The further studies in decrementless conduction. Tokyo, 1926.
KRONECKER, H. Arch. f. Physiol., p. 263, 1883.
LUCAS, K. J. Physiol. **36:** 253, 1907.
MARTIN, E. G. This Journal **11:** 370, 1904.
MINES, G. R. J. Physiol. **37:** 408, 1908.
RINGER, S. J. Physiol. **8:** 289 1887.
RÖSSLER, R. Ztschr. f. Biol. **81:** 299, 1924.
SAITO, S. Ztschr. f. Biol **48:** 340, 1906.

ON THE INFLUENCE OF PEPTONES AND CERTAIN EXTRACTS OF SMALL INTESTINE UPON THE SECRETION OF SUCCUS ENTERICUS

E. S. NASSET AND H. B. PIERCE

From the Department of Vital Economics, University of Rochester, Rochester, N. Y.

Received for publication June 28, 1935

It was demonstrated recently that the secretion of the jejunum in the dog is subject to a humoral control (Nasset, Pierce and Murlin, 1935). Florey and Harding (1935) have shown the same to hold true for the duodenum in the cat and in the rabbit. In both investigations, the influence of extrinsic nerves was ruled out by the use of the auto-transplantation technic devised by Ivy and Farrell (1925). The results confirm a belief which has been held by various investigators at different times. The earlier evidence in support of this belief has been summarized by Babkin (1928).

Little is known concerning the substances responsible for accelerating the secretion of intestinal juice. Delezenne and Frouin (1904) injected, intravenously, boiled and neutralized acid extracts of the intestine, and obtained an abundant secretion of duodenal juice from dogs with Thiry fistulae. Their conclusion was that the stimuli for duodenal, pancreatic and biliary secretion were identical. Frouin (1905) injected the clear portion of succus entericus itself and obtained an increased secretion from a duodenal Thiry fistula. From the fact that this treatment had no effect upon pancreatic secretion he concluded that the active substance could not be secretin. This agrees with some observations made in this laboratory. Ågren (1934) using anesthetized cats, reports that crystalline secretin increases the secretion of fluid and alkali in the small intestine but has no effect upon enzyme production. Botazzi and Gabriele (1905) were successful in augmenting the production of intestinal juice by the intravenous administration of an extract of mucosa. Mironescu (1910) found that extracts of the gastro-intestinal tract, from esophagus to colon, were active when given subcutaneously, whereas brain, liver and kidney extracts were inactive.

An extensive study of the activity of Witte's peptone as a secretagogue was made by Popielski (1909). On injecting solutions of Witte's peptone intravenously, he found an increase in the rate of flow of saliva, bile, pancreatic and gastric juice. In one experiment, similar injections

did not cause a greater production of intestinal juice in a Thiry loop of a dog.

The relatively slow progress in this field has been due, in part, to the lack of a suitable acute method. In the pancreas, the salivary glands and in the liver where the ducts may be cannulated, the work of assaying numerous preparations has been simple and rapid. Nasset and Parry (1934) proposed an acute method for the study of changes in the rate of intestinal secretion. It is believed that with the aid of this method better progress will be made in the elucidation of the nature of the secretagogues or hormones which play a rôle in the control of intestinal secretion.

METHODS. 1. *Test animals.* a. *Chronic.* Two dogs bearing isolated and denervated jejunal transplants were used in determining the activity of the peptone preparation. These animals have been in the colony for some time and served in experiments establishing the humoral control of intestinal secretion. The details of operative technic and the history of these animals (nos. 6 and 8) appear in previous papers (Pierce, Nasset and Murlin, 1935; Nasset, Pierce and Murlin, 1935).

b. *Acute.* The dogs (18 to 35 kgm.) were taken from stock, given a vermifuge 2 days before experiment and fasted the last 24 hours. Amytal and Dial[1] anesthesia were used.

Essentially the principle of the acute method is to fill a segment of intestine, about 15 cm. long, with saline and reduce the intraintestinal pressure until the rate of secretion of fluid just exceeds its absorption. This usually occurs at about −15 cm. of saline below atmospheric pressure. Collapse of the segment is prevented and a relatively constant volume is maintained by the insertion of a helical wire coil. It is important to maintain the normal temperature of the intestine and this is done with a moist chamber fitted over the dog's belly, enclosing the exposed intestinal segment which rests just outside of the abdominal wall, on a small platform. The procedure is fully described and illustrated in an earlier paper (Nasset and Parry, 1934).

A recent modification is the introduction of a drop recorder to replace the burette formerly attached to the open end of the system and which served to indicate changes in volume. Changes in secretory rate are now graphically recorded along with blood pressure, time, etc. The method is convenient in that the basal rate of secretion can be altered at will by changing the intraintestinal pressure. It is important to keep in mind that the results obtained do not indicate the magnitude of the total secretion. Because of the constant reabsorption of fluid, all that can be detected by this method is the deviation from the control or resting rate. It should be emphasized that this method permits the detection of small

[1] We are indebted to Dr. C. C. Haskell of the Ciba Company for the Dial used in this work.

changes in the rate of secretion and in short intervals of time. In this respect, it compares favorably with the acute methods used in the study of pancreatic, salivary or biliary secretion.

2. *Preparation of extracts.* a. *Peptone.* Witte's peptone was extracted with 70 per cent ethyl alcohol (3 parts 95 per cent plus 1 part water), the alcohol evaporated off on the steam bath and the residue finally dried in vacuo. This substance was readily soluble in water, gave no precipitate on addition of trichloracetic acid and maintained most of its secretagogue activity after autoclaving at 122° to 124° for 20 minutes. Any vasodepressor activity in solutions of Witte's peptone, or of the 70 per cent alcohol soluble portion (peptone "B") was readily removed by adsorption on Fuller's earth. Peptone "B" has been given intravenously in both chronic and acute experiments with similar results, i.e., an increased production of intestinal juice.

b. *Small intestine.* This organ from dogs and swine was extracted with 70 per cent acid ethyl alcohol as follows: The intestine was slit open lengthwise, washed, the mucosa scraped off and the scrapings extracted or the intestine was simply turned inside out, washed and extracted. To each 100 grams of tissue were added 300 cc. of 95 per cent ethyl alcohol, 25 cc. of water and 1 cc. of concentrated HCl. Figuring the water content of the tissue at approximately 75 per cent of its moist weight, the final concentration of alcohol should be about 70 per cent. Extractions were carried out at room temperatures for 24 to 48 hours. The alcohol in the clear, tan-colored filtered extract was distilled off at reduced pressure and a temperature of about 50°C. When the extract had been reduced to one-fifth to one-seventh of its volume, a considerable amount of grayish material separated out. Water was added to redissolve it in part, but the aqueous solution remained opalescent. Clear solutions were obtained by adjusting the pH to 5.8 to 6.0, at which point most of the suspended colloidal material precipitated out. The pH of the extract was about 4.0.

This method is not given as the only method by which potent extracts may be obtained. Indeed, extraction by 0.9 per cent NaCl, or 0.4 per cent HCl both have given good results, but in our hands have extracted more extraneous material than does the alcohol.

Results. *Peptones.* The earliest experiments using extracts of the mucosa showed the presence of a potent secretagogue and invariably also the presence of the so-called "vaso-dilatins" common to all simple extracts of the intestine. It seemed desirable to dissociate these two classes of substances. Witte's peptone given intravenously was known to cause a vascular response similar in nature and duration to that obtained with the intestinal extracts and, therefore, it was thought that the administration of the former might shed some light on the relation of intestinal

secretion to vasodilatation. The first trial of Witte's peptone, using the acute method, was at once disappointing and surprising in that the expected vascular response was accompanied by a copious secretion. Rates as high as 1 cc. per minute for 30 minutes were observed in a 15 cm. loop of jejunum during continuous injection of 90 cc. of 5 per cent Witte's peptone over the same period of time. There was considerable recovery from the initial vasodilatation before the injection had been completed. The volume of juice secreted was over twice the volume of the loop of intestine being used.

With the development of peptone "B" (70 per cent alcohol soluble) it was found that vasodepressor effects could be eliminated with retention of the secretagogue activity. Doses of 200 to 500 mgm. of this substance gave an excellent secretory response. Peptone "B" was given intrave-

TABLE 1

Effect of intravenous injection of peptone "B" on the secretion of intestinal juice from denervated jejunal transplants

DATE	DOG NUMBER	DOSE	VOLUME OF JUICE PER HOUR		SUCRASE ACTIVITY—INVERT SUGAR FORMED		PEPTIDASE ACTIVITY—AMINO N FORMED	
			Control	Experimental	Control	Experimental	Control	Experimental
1926		*mgm.*	*cc.*	*cc.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1/9	6	400	4.8	14.2	1,813	5,609	153	665
6/3	6	500	2.4	8.6	845	3,939	205	783
6/5	6	250	3.7	8.6	320	544	276	441
1/9	8	400	2.3	11.4	367	3,215	54	485
6/3	8	450	3.8	12.0	897	1,380	247	719
6/5	8	200	2.9	6.4	58	228	188	477

nously 4 times to two of the operated dogs with no ill effects and with a very great augmentation of the production of both fluid and enzymes (sucrase and peptidase). The results of three of these experiments are given in table 1. (The 4th experiment was not included because the control enzyme determinations were lost.) These injections were always given on fasting days at the end of a regular 7-hour collection period when the production of both fluid and enzyme is most likely to be at a low point. Thus the results first obtained with the acute method were corroborated by experiments on the chronic dogs. Still and Barlow (1927) found that the intravenous injection of Witte's peptone resulted in a large increase in the secretion of trypsinogen in acute experiments. However, they were inclined to attribute this effect to the vasodilatins present in the preparation.

It is of interest to note that 5 peptone preparations obtained from the

Digestive Ferments Company[2] were almost wholly inactive as secretagogues, although one of them produced a more profound vasodilatation than did any of the Witte's peptones.

Witte's and Difco peptones were given in 50 gram portions by stomach tube to the two chronic dogs with the results indicated in figure 1. It can be seen that this method of administration also is effective in bringing about a decided increase in the volume of juice produced in the denervated

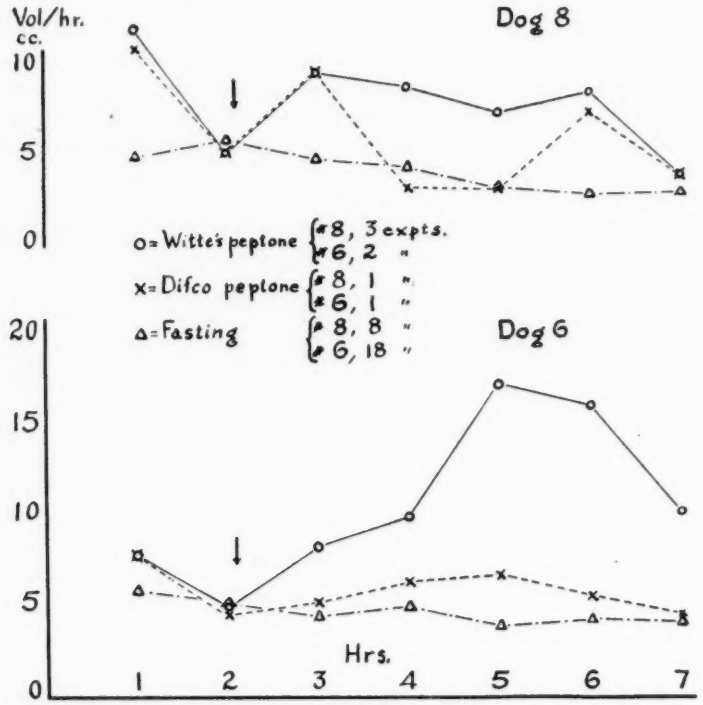


Fig. 1. The effect of peptone feeding upon secretion of intestinal juice from denervated jejunal transplants. Peptone was given at the times indicated by the arrows.

jejunal loops. This result presents an apparent paradox because the secretagogue activity of peptone "B" at least is entirely destroyed by digestion in vitro by commercial erepsin or trypsin (fig. 2B). One possibility is that the mere presence of the substance in the gastro-intestinal

[2] Two of these, "Difco-Bacto" and "Bacto-Protone," were on hand in the laboratory; the other three were especially prepared for us through the courtesy of Mr. H. G. Dunham of the Digestive Ferments Co.

tract causes the production of a hormone which excites the isolated loops. However, this does not explain the prompt response observed upon intravenous administration. Another possibility is that enough of the secretagogue is absorbed unchanged to bring about the results observed after the feeding experiments. In any case, the indications are that the active principle may be a peptide. Martens (1931) demonstrated the passage of peptides through the intestinal wall by chemical analyses of the portal blood.

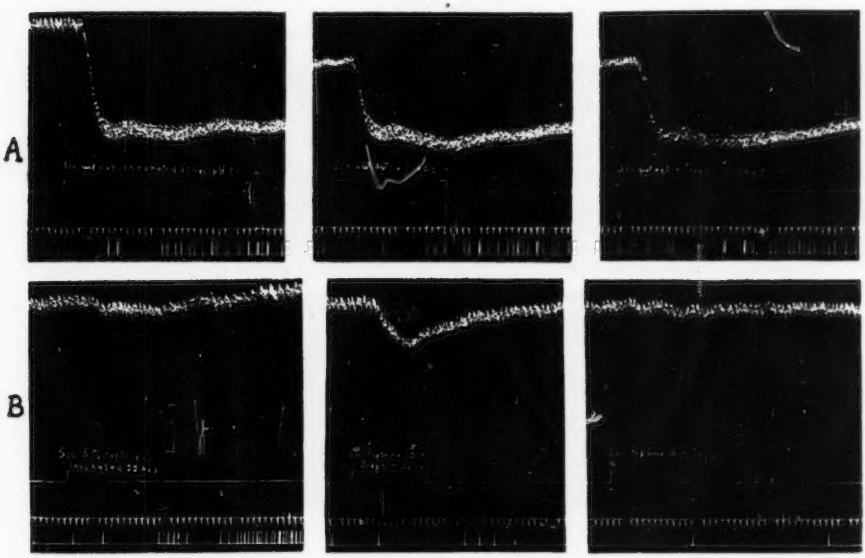


Fig. 2. Top line is blood pressure, 2nd line signal, 3rd line time in 5-second intervals, and the bottom line drops of secretion.

A shows the resistance of the secretagogue of intestinal extract to ereptic and tryptic digestion in vitro.

B shows the destruction of the secretagogue of Witte's peptone by erepsin and trypsin in vitro.

Intestinal extracts. In figure 2A are shown typical results from the intravenous injection of a hog gut extract which contained 1.88 per cent of organic solids. Thus the administration of 94 mgm. of crude material increased the rate of secretion by approximately 0.5 cc. per minute for several minutes (18 drops = 1 cc.). These data show further that commercial erepsin or trypsin are unable to destroy the secretory activity of the crude extract.

It has not been possible as yet to obtain a vasodepressor-free secretagogue from extracts of the intestine. Fuller's earth has failed to remove

the objectionable substances which evidently are different from those found in the peptones. Recently, however, in two acute experiments it was found possible to dissociate the secretory and the vasodepressor activities of an extract of intestine by boiling in alkali. The former disappeared whereas the latter persisted almost unchanged after boiling 2 to 6 minutes in 0.35 normal NaOH (fig. 3A). This sort of dissociation is not so satisfactory as the converse but certainly indicates that there are at least two different substances concerned. It may be recalled that the secretin theory of Bayliss and Starling found acceptance for about a quarter of a century before the advent of "vasodilatin"-free secretin.

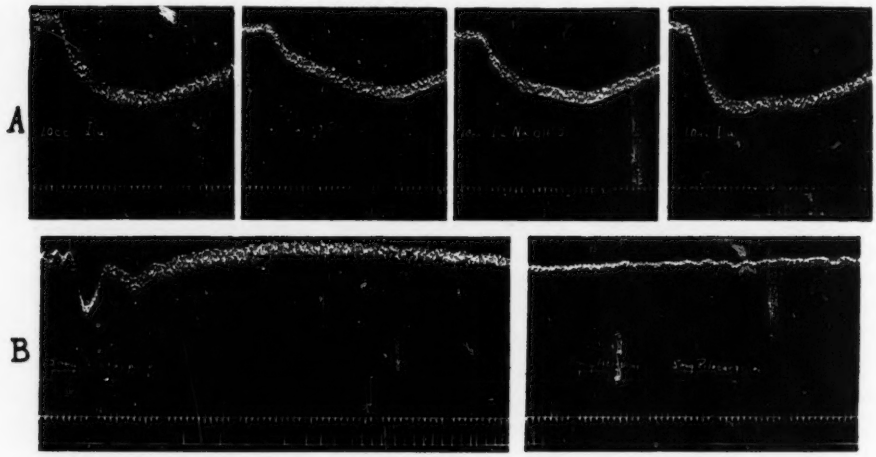


Fig. 3. A, showing the dissociation of vaso-depressors and secretagogue in an intestinal extract, containing 42 mgm. of organic solids.

B. Typical results of pilocarpine and atropine upon intestinal secretion as determined by the acute method.

Since the secretion-promoting substance has not yet been obtained free from vasodepressors, and other impurities, it has been deemed unwise to inject the extracts into the operated dogs. In the acute experiments, the increased secretion of fluid following injections of the preparations described has been obtained repeatedly. It was desired to know whether the secretion of typical enzymes followed a parallel course. In figure 4 are shown the results of an experiment done to answer this question. In this experiment 55 cc. of saline, at loop temperature, was siphoned through the loop of intestine, washing out the contents every 15 minutes. Every other period served as a control. To one 25 cc. portion of the contents was added 25 cc. of 2 per cent sucrose solution, and to

another was added 25 cc. of 5 per cent peptone solution. After 24 hours' incubation at 38°C., the amount of invert sugar formed was determined by Bertrand's method and the amount of amino nitrogen liberated by formol titration. It is shown very clearly that at least invertase and peptidase are produced in larger quantities whenever the secretion of fluid is increased. The experiment also confirms the conclusion (Nasset and Parry, 1934) that even though the "apparent secretion" may be nil, as judged by the fluid contents of the loop, there is still a true secretion going on as evidenced by the production of enzymes.

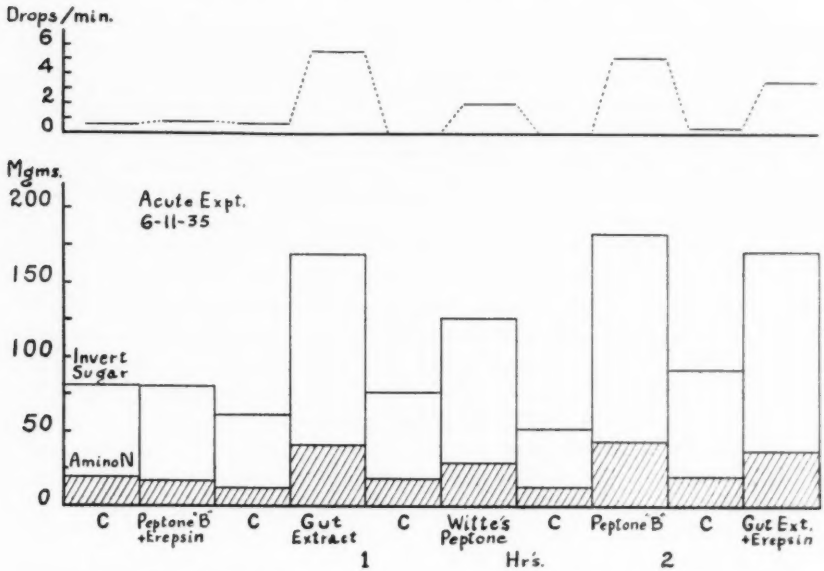


Fig. 4. Showing the relation of fluid and enzyme secretion in an acute experiment. All periods 15 minutes. C = control periods, 250 mgm. of peptone were given and 94 mgm. of organic solids in the gut extract.

No systematic study of drugs has been made using the acute method. A few of the more commonly used ones have been tried occasionally. Figure 3B shows the response to pilocarpine in one experiment and the characteristic action of atropine. The latter may decrease the response to intestinal extracts but cannot abolish it. Epinephrine in doses of 0.05 to 0.10 mgm. causes a transient increased secretion. Histamine phosphate also, in doses of 0.5 to 1.0 mgm. causes some stimulation. This latter finding is in agreement with the results of Berndt and Ravdin (1934) on chronic dogs.

DISCUSSION. The elucidation of the secretagogue activity of Witte's peptone presents some puzzling aspects. From the results of the digestion studies, one might suspect that a peptide was responsible. Furthermore, since 5 other peptones were found inactive, it appears that a specific peptide may be concerned. Presumably Witte's peptone is manufactured by partially digesting blood fibrin. Three of the other peptones tested (Digestive Ferments Co.) were specially prepared from well washed blood fibrin (beef) by partial digestion, arrested at various stages. The difference may lie in the manner of digestion or the thoroughness of washing the fibrin.

The fact that peptone "B" is an effective secretagogue, given either by mouth or by vein, is interesting in light of its digestibility in vitro by enzymes which are typically found in the gastro-intestinal tract. Obviously, the presence in the blood of the end products of this digestion cannot be the condition requisite for the stimulation of the intestinal glands (fig. 2B). Conceivably intravenous injection of the active substance could result in its passage from blood to lumen where it might exert its influence. However, the latent period after intravenous injection is usually less than one minute in both chronic and acute experiments, a fact which makes the latter hypothesis improbable.

The most likely explanation of the effect following oral administration is that a portion of the active substance escapes digestion and is absorbed unchanged. The stimulus must act directly upon the intestinal glands because the chronic dogs used had jejunal transplants completely isolated from their mesenteric nerves and blood vessels.

From the experiments with the extracts it appears that the mucosa of the small intestine of dogs and swine contains a substance which when given by vein results in an increased secretion of both fluid and enzymes by the small intestine (jejunum). Furthermore, it appears that the secretagogue and vasodepressor substances in such extracts are separate entities. Although the results of these experiments have been obtained with acute animal preparations, it seems clear from the peptone experiments that they will be corroborated in the chronic preparations whenever the extracts become safe for intravenous use.

The crude secretagogues from the two sources are similar in solubility and thermostability but are quite different in their resistance to ereptic and tryptic digestion. Decision as to whether the active principle in the intestinal extracts is a hormone must await further work.

SUMMARY

1. Witte's peptone contains a potent secretagogue which, when given either by mouth or by vein, acts upon the glands of the jejunum. This active material is extractable, free of vasodepressor substances, with 70

per cent ethyl alcohol. It is digested by erepsin or trypsin. It is thermostable.

2. Five other peptones showed little or no secretagogue activity although one of them caused a greater vasodilatation than did Witte's peptone.

3. The substance from Witte's peptone when given intravenously to dogs with denervated jejunal transplants, may cause a 3- to 5-fold increase in the volume of juice secreted and a 2- to 8-fold increase in enzyme production.

4. Small intestines from dogs and swine, extracted with acidulated 70 per cent ethyl alcohol, yield a crude extract which stimulates intestinal secretion when given by vein in acute experiments. Both fluid and enzyme components are increased. The substance is thermostable and resists ereptic and tryptic digestion.

5. The vasodepressors and the secretion-promoting substance of these extracts are not identical because the latter is destroyed by boiling in alkali whereas the former are scarcely affected.

6. A slight modification of a previously described acute method makes it possible to record graphically changes in the rate of intestinal secretion over relatively short periods of time.

REFERENCES

ÅGREN, G. Skand. Arch. Physiol. **70:** 10, 1934.

BABKIN, B. P. Die aüssere Sekretion die Verdauungsdrüsen, zweite Auflage, Berlin, 1928.

BERNDT, A. L. AND I. S. RAVDIN. This Journal **109:** 587, 1934.

BOTAZZI, F. AND L. GABRIELE. Arch. internat. de physiol. **3:** 156, 1905.

DELEZENNE, C. AND A. FROUIN. Compt. rend. Soc. Biol. **56:** 319, 1904.

FLOREY, H. W. AND H. E. HARDING. Proc. Roy. Soc. **117B:** 68, 1935.

FROUIN, A. Compt. rend. Soc. Biol. **57:** 702, 1905.

IVY, A. C. AND J. I. FARRELL. This Journal **74:** 639, 1925.

MARTENS, M. R. Bull. Soc. Chim. Biol. **13:** 1187, 1931.

MIRONESCU, T. (Quoted from BABKIN, 1928) 1910.

NASSET, E. S. AND A. A. PARRY. This Journal **109:** 614, 1934.

NASSET, E. S., H. B. PIERCE AND J. R. MURLIN. This Journal **111:** 145, 1935.

PIERCE, H. B., E. S. NASSET AND J. R. MURLIN. J. Biol. Chem. **108:** 239, 1935.

POPIELSKI, L. Pflüger's Arch. **126:** 483, 1909.

STILL, E. U. AND O. W. BARLOW. This Journal **81:** 341, 1927.

FURTHER EVIDENCE FOR THE PRIMACY OF POLYURIA IN DIABETES INSIPIDUS

CURT P. RICHTER AND JOHN F. ECKERT

From the Psychobiological Laboratory, Johns Hopkins Hospital, Baltimore, Md.

Received for publication June 28, 1935

It was found that rats with acute or chronic diabetes insipidus showed a persistent polyuria even when they were totally deprived of water (Richter, 1935). So marked was the fluid imbalance that some of the animals lost as much as one-sixth of their body weight within the first ten hours. This fact and the observation that polyuria preceded polydipsia after the experimental production of diabetes insipidus were taken as definite evidence for the primacy of polyuria in this condition.

Further evidence for the same conclusion was obtained in the present experiments in which it was found, conversely, that blockage of the urine outflow in rats with diabetes insipidus completely abolished the polydipsia.

METHODS. The technique used in these experiments has already been described in detail (Richter, 1935). In brief, the rats were kept in individual cages $4\frac{1}{2} \times 4\frac{1}{2} \times 8$ inches with a graduated water bottle and a wire mesh bottom with a trough beneath for the collection of urine. No food was given.

Hourly readings of urine output and water intake were made during the first twelve hours and a single record was taken at the end of eighteen to twenty hours.

The diabetes insipidus was produced by partial hypophysectomy (removal of the posterior lobe and part of the anterior lobe); the urine output was blocked by ligation of both ureters about six hours after the hypophysectomy when the polydipsia had reached a plateau.

RESULTS. A typical record is presented in figure 1 A showing the polydipsia produced in the rat by hypophysectomy. It will be seen that the water intake was zero for the first two hours after the operation and then increased very rapidly reaching a high level during the fifth hour which was maintained fairly constantly for twelve hours after the operation. Thirteen other experimental animals gave similar results.

It will be seen in figure 1 B that ligation of the ureters at the end of the sixth hour, after the definite establishment of a constant high level of water intake, caused almost immediately the disappearance of the poly-

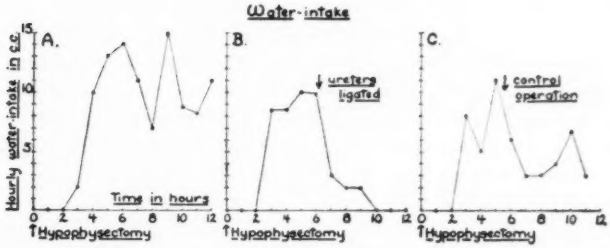


Fig. 1. Graph showing polydipsia produced by partial removal of the pituitary gland and the effect of ligation of ureters.

Ordinates: Hourly water intake in cubic centimeters.

Abscissae: Time in hours.

A. Typical water intake record showing effect of hypophysectomy alone. B. Typical water intake record showing effect of hypophysectomy followed by ligation of ureters at the end of sixth hour. C. Typical water intake record showing effect of control operation (abdominal incision and manipulation of intestines without ligation of ureters).

TABLE 1

ANIMALS WITH DIABETES INSIPIDUS WITH URETERAL LIGATION			ANIMALS WITH DIABETES INSIPIDUS WITHOUT URETERAL LIGATION		
Rat number	Average water intake in cubic centimeters for two hour period before ligation (fifth and sixth hours after hypophysectomy)	Average water intake in cubic centimeters for six hour period after ligation (seventh to twelfth hours after hypophysectomy)	Rat number	Average water intake in cubic centimeters for period of fifth and sixth hours after hypophysectomy	Average water intake in cubic centimeters for period from seventh to twelfth hours after hypophysectomy
1	9.0	0.7	15	8.5	7.6
2	4.5	1.0	16	8.0	7.6
3	4.5	2.0	17	5.0	5.3
4	10.0	1.0	18	10.5	10.6
5	8.0	0.8	19	12.5	7.6
6	2.5	0.5	20	4.0	4.6
7	4.0	0.1	21	7.0	4.0
8	8.0	0.0	22	2.0	3.0
9	11.5	0.8	23	6.5	3.0
10	6.5	1.0	24	4.0	4.0
11	12.5	0.3	25	5.0	4.1
12	5.0	0.3	26	6.0	4.0
13	9.0	0.7	27	7.0	6.0
14	7.5	1.0	28	7.5	5.0
Total average..	**7.32**	**0.73**		**6.67**	**5.43**

dipsia. The water intake diminished in the next four hours from a level of 10 cc. to zero. Similar records were obtained from the other thirteen animals.

Definite evidence was obtained that the ligation operation itself was not responsible for the disappearance of the polydipsia. Control operations in animals with diabetes insipidus, consisting of incision of the abdominal wall and manipulation of the intestines without ligation of the ureters, did not eliminate the polydipsia. See figure 1 C. The temporary depression in the water intake was due undoubtedly to the ether anesthesia. Similar results were obtained in eight other animals.

The results of these experiments are summarized in table 1. The average nourly water intake for fourteen animals with diabetes insipidus and with ligation of the ureters dropped from 7.32 cc. per hour for the two hour period immediately preceding the ligation (5th and 6th hours after hypophysectomy), to 0.73 cc. per hour or ninety per cent for the six hour period immediately following (7th to 12th hour after hypophysectomy). However, it is apparent that the water intake would have been maintained at a high level in these animals had the ureters not been ligated. The average hourly water intake for fourteen control animals with diabetes insipidus without ureter ligation was 6.67 cc. per hour for the 5th and 6th hours after hypophysectomy and 5.43 cc. per hour for the following six hours (7th to 12th hour after hypophysectomy).

The absence of the polydipsia following ligation of the ureters was not due to a general incapacitation of the animals. A control group of eight rats with ureters ligated, kept under normal conditions, with access to food and water, ate, drank and were normally active for the first eighteen hours and survived as long as five days. Two out of sixteen animals even showed a mild polydipsia, drinking as much as 44 cc. during the eighteen hours after ligation of the ureters, a result which may be attributed to a need of water for the dilution of the waste products accumulated as a result of the blockage of the urine outflow.

This evidence, in agreement with the results reported previously, demonstrates that polydipsia is a secondary factor to polyuria in diabetes insipidus. The possible significance of this fact and its relation to the etiology of diabetes insipidus was discussed in detail in the previous paper.

SUMMARY

1. Polydipsia produced in fourteen rats by partial hypophysectomy was eliminated by ligation of the ureters.

2. Control operations with ether anesthesia and intra-abdominal manipulation without ligation of the ureters, in eight animals with diabetes insipidus, did not eliminate the polydipsia.

3. Control ligation of ureters in normal rats did not lower the water intake, but actually produced a definite increase in two animals during the first eighteen hours.

4. This evidence is in agreement with the previous conclusion that the increased thirst is secondary to the increased loss of urine in the diabetes insipidus syndrome.

REFERENCE

RICHTER, C. P. This Journal **112**: 481, 1935.

THE EFFECT OF DIET ON THE HEMOGLOBIN CONCENTRATION OF THE BLOOD

L. N. ELLIS AND O. A. BESSEY

From the Department of Chemistry, Columbia University

Received for publication June 28, 1935

Attempts to obtain a standard value for the hemoglobin concentration of the blood of normal men and women have produced a range of figures (1). Similar attempts in the laboratory, using rats, have likewise resulted in a divergence of results (4) (5) (9). While some of the disagreement can be explained by the different methods of determining hemoglobin, nevertheless other factors must be concerned (2).

In 1931 Mitchell (3) suggested that the practice in the different laboratories of inbreeding for many generations on the same diet might produce differences in hemoglobin concentration as well as differences in rate of growth. While Orten and Smith (5) found no such effect of diet, Drabkin and Fitz-Hugh (4) in comparing two groups of Wistar rats on different stock rations found a noticeable difference in concentration of hemoglobin.

A more thorough investigation of the influence of diet on the hemoglobin content of the blood was undertaken in hopes of helping to clarify the situation. There was available a colony of rats the inbreeding of which for many generations made their heredity an uncomplicating factor. Any differences found, therefore, were to be attributed mainly to dietary influence.

PROCEDURE. Normal rats from five diets were investigated. With the exception of one, the diets were simple mixtures of whole wheat and whole milk powder with some slight modifications. Diets A and B of Sherman and Campbell (6) were selected because of the extensive work which had been reported with respect to these diets. The other diets consisted of diet B with the addition of raw beef and fresh string beans (here designated as diet B+meat+beans), diet B with the substitution of casein for some of the whole wheat (diet B+casein), and a diet containing a mixture of foodstuffs representative of human dietaries (diet H).

Two age levels were selected for the study. First, a year of age was chosen to study the influence of diet on the maintenance of hemoglobin in conjunction with adult activities. And second, a month of age was taken to study the effect of these same diets upon the regeneration of hemoglobin during recovery from anemia of infancy.

The Newcomer method for determination of blood hemoglobin was used (7) and the grams per 100 cc. obtained by use of a Newcomer disc (purchased 1934) in a Klett colorimeter. The blood was drawn from the tip of the tail which had been previously warmed in water to stimulate the peripheral circulation. Single samples were taken at a month of age when the blood volume was small, but duplicate samples were always taken at a year of age as checks on the reproducibility of sampling. To reduce the interference of exercise, pregnancy, or of slight disorders, check determinations within a period of a week to a month were made on the adults.

TABLE 1

Hemoglobin content of rat's blood

One month of age

DIET	NO. RATS AND SEX	AV. WT.	GM. Hb / 100 CC. ± S.D. $_m$	MGM. Fe / 100 GM.	GM. PROTEIN / 100 GM.
		grams			
H	8♂	47	9.2 ± 0.5	2.89	17.9
	12♀	45	10.3 ± 0.2	2.89	17.9
B + meat + beans	12♂	51	10.4 ± 0.3		
	11♀	46	11.3 ± 0.4		
B	12♂	48	11.7 ± 0.3	3.12	15.5
	12♀	49	11.8 ± 0.4	3.12	15.5
B + casein	12♂	56	11.5 ± 0.4	3.64	25.3
	13♀	51	12.0 ± 0.4	3.64	25.3
A	12♂	43	12.6 ± 0.3	3.68	14.0
	12♀	44	12.6 ± 0.2	3.68	14.0

The iron content of the diets was determined by Elvehjem's modification of the Kennedy method (8). Because of the large proportion of whole wheat in the diets, the supply of iron was adequate and readily available for utilization.

EXPERIMENTAL RESULTS. At a month of age a minimum value of 9.2 grams was obtained for diet H from which the hemoglobin concentration increased for diets B+meat+beans and B to a maximum value of 12.6 grams for diet A. The differences were more clear cut with the males where, with the exception of the very similar results for diets B and B+casein, the ratio of the difference to the standard deviation of the difference, being 2 to 3, indicated a reasonable probability of a true difference. With both males and females, however, the increase of hemoglobin for diets A and B above that for diet H was certainly significant.

A correlation was found between the hemoglobin concentration and the iron content of the diet, a 40 per cent increase of hemoglobin being observed for a 25 per cent increase of iron. The hemoglobin concentration was independent of weight as evidenced by both the maximum and minimum values being obtained with the lowest weights. No study of the concentration of copper was made since the presence of large quantities of wheat in the diets insured ample amounts of that element.

The value of 12.6 grams of hemoglobin per 100 cc. is as high a figure as reported in the literature for this age, Williamson and Ets (9) finding 12.62 and Drabkin and Fitz-Hugh (4) 13.0. Diet A is therefore very

TABLE 2

Hemoglobin content of rat's blood

One year of age

DIET	NO. RATS AND SEX	AV. WT.	$\frac{\text{GM. Hb}}{100 \text{ CC.}} \pm \text{S.D.}_m$	$\frac{\text{MGM. Fe}}{100 \text{ GM.}}$	$\frac{\text{GM. PROTEIN}}{100 \text{ GM.}}$
		grams			
H	8♂	387	15.3 ± 0.3	2.89	17.9
	8♀	222	14.0 ± 0.4	2.89	17.9
B	8♂	343	15.3 ± 0.4	3.12	15.5
	9♀	238	14.8 ± 0.2	3.12	15.5
A	10♂	392	15.7 ± 0.4	3.68	14.0
	8♀	249	14.4 ± 0.3	3.68	14.0
B + meat + beans	10♂	399	16.2 ± 0.3	2.86	14.8
	12♀	262	15.6 ± 0.2	2.86	14.8
B + casein	7♂	409	15.8 ± 0.4	3.64	25.3
	10♀	256	15.9 ± 0.3	3.64	25.3

efficacious for the regeneration of hemoglobin following the anemia of infancy.

At a year of age only small differences in hemoglobin concentration were observed, and not in the same relationship as at a month of age. The diets fell roughly into two groups. That is, the hemoglobin concentration on diets H, A and B were about 15.3 grams for the males and 14.4 grams for the females. Significantly higher concentrations of 15.7 grams were found for the females of diets B+meat+beans and B+casein. The males on the two diets also showed slightly, but possibly not significantly, higher hemoglobin concentrations.

Hemoglobin determinations were made for diets B and B+meat+beans at 60 and 90 days to ascertain when the latter diet became superior to diet B. At 60 days of age both the males and females on diet B + meat + beans

had not only equalled but surpassed those on diet B in hemoglobin concentration. For example, the females had increased their hemoglobin by 3.5 grams per 100 cc. as against 1.8 grams on diet B. At 90 days of age the same concentration as measured at a year of age was reached.

The two groups of figures obtained here represent roughly the range of normal values for adult rats reported by various workers (Drabkin and Fitz Hugh, 18.4; Mitchell and Miller, 16.2; Wills and Mehta, 14.1; Williamson and Ets, 14.2; and Enzman, 14.4 grams). It therefore became evident that some of the variance in determination of a normal standard might have been due to the use of different diets and that no standard can be set until the influence of diet has been more thoroughly investigated.

The superiority of hemoglobin concentration of the females on diet B+casein may well be due to the presence of an abundance of such factors as protein and iron. The reason for the superiority of diet B+meat+beans is less evident and merits further study. The attainment of greater adult weight on these two diets is partial evidence of the superiority of these diets in other respects.

SUMMARY

Hemoglobin determinations on rats were made at one month and one year of age to study the influence of five diets. At one month of age the hemoglobin concentration increased along with the iron content of the ration. A maximum value of 12.6 grams per 100 cc. was obtained. At one year of age the hemoglobin concentrations were divided roughly into two groups and were independent of the iron content of the diet. With three of the diets values of 15.3 grams for the males and 14.4 grams for the females were obtained and for the other two diets a concentration of 15.7 for the females.

REFERENCES

(1) Haden, R. L. Am. J. Clin. Path. **3:** 85, 1933.
(2) Robscheit-Robbins, F. S. Physiol. Rev. **9:** 666, 1929.
(3) Mitchell, H. S. and L. Miller. This Journal **98:** 311, 1931.
(4) Drabkin, D. L. and J. T. Fitz-Hugh, Jr. This Journal **108:** 61, 1934.
(5) Orten, J. M. and A. H. Smith. This Journal **108:** 66, 1934.
(6) Sherman, H. C. and H. L. Campbell. J. Biol. Chem. **60:** 5, 1924.
(7) Newcomer, H. S. J. Biol. Chem. **37:** 465, 1919.
(8) Elvehjem, C. A. J. Biol. Chem. **86:** 466, 1930.
(9) Williamson, C. S. and H. N. Ets. This Journal **77:** 480, 1926.
(10) Enzman, E. V. This Journal **108:** 373, 1934.
(11) Wills, L. and M. M. Mehta. Ind. J. Med. Research **18:** 307, 1930.

EFFECT OF PHYSICAL TRAINING ON BLOOD VOLUME, HEMOGLOBIN, ALKALI RESERVE AND OSMOTIC RESISTANCE OF ERYTHROCYTES[1]

JOHN E. DAVIS AND NATHAN BREWER

From the Department of Physiology, University of Chicago

Received for publication June 14, 1935

That previously confined dogs, when exercised on treadmills for a few days, undergo a loss of blood volume, erythrocytes and hemoglobin has been shown by Broun (1, 2). This destruction he attributes to a process of mechanical fragmentation of cells which was earlier advocated by Rous and Robertson (3) as a normal mode of blood destruction. McMaster, Broun and Rous (4), using biliary fistula dogs, showed that an increased bilirubin output accompanied hemoglobin reduction during a few days of treadmill exercise. Thörner (5, 6) has reported an increased osmotic resistance of erythrocytes in man and dog after physical training. Hastings (7) has shown that in vivo hemolysis occurs in the serum of untrained dogs after exercise. Broun's dogs showed recovery of erythrocytes and hemoglobin with continued exercise, and of blood volume after three weeks' rest. Anemic anoxemia has been suggested by Steinhaus (8) as the bone marrow stimulant in this recovery process. He attributes the high erythrocyte osmotic resistance of Thörner's trained dogs to a weeding out of older, less resistant corpuscles by the fragmentation process which is augmented by the higher circulatory rates of exercise.

The present work was undertaken to determine whether or not prolonged physical training will induce an increase of blood volume and erythrocytes and, if possible, to shed light on the stimulus to blood formation and the mode of blood destruction in exercise.

PROCEDURE Five dogs were confined in cages for two to three months without exercise. During this period and throughout the training and post-training periods that followed, the dogs were fed daily a constant mixed diet of 200 grams beef lungs, 200 grams of beef liver, and 200 grams bread. Two dogs were exercised by swimming for two hours daily in water at 30°C.; and three dogs were made to run on a treadmill on 25 per cent grade for a distance of six miles daily. The exercise periods varied in length from four to nine weeks, and post-exercise observations were

[1] The present investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

continued for four to six weeks thereafter. The two forms of exercise were adopted to cover possible effects on the blood and of pH variations, which according to Rice and Steinhaus (9) vary with the body temperature. Rectal temperatures in our dogs were increased by about 1°C. after running, and decreased about 2°C. after swimming.

Body weights of the animals did not change by more than ½ kgm. throughout the experiments.

TABLE 1

Osmotic resistance of erythrocytes. Concentrations of sodium chloride in which 50 per cent of the hemoglobin is laked

TIME OF DETERMINATION	DOG 5	DOG 4	DOG 3
	per cent	*per cent*	*per cent*
23 days before exercise	0.427		
20 days before exercise	0.424		
15 days before exercise	0.432	0.432	0.456
11 days before exercise	0.434	0.432	0.462
9 days before exercise	0.430		0.458
6 days before exercise	0.434		
3 days before exercise	0.432		
Pre-exercise average	0.430	0.432	0.458
After 6 days' exercise		0.433	0.440
After 13 days' exercise	0.440	0.400	
After 16 days' exercise	0.444		
After 20 days' exercise	0.443	0.415	0.440
After 27 days' exercise	0.440		0.443
After 36 days' exercise		0.411	0.444
After 41 days' exercise		0.397	0.447
After 48 days' exercise		0.400	0.444
Exercise average	0.442	0.405	0.443
28 days after cessation exercise		0.415	0.456
38 days after cessation exercise		0.415	0.455
Post-exercise average		0.415	0.455

Dogs were fed during the training period at the conclusion of the daily exercise. All blood determinations were made under basal conditions of the animal at least eighteen hours after previous exercise and food. The following blood determinations were made: Blood volume by the carbon monoxide inhalation method of Chang and Harrop (10), using the blood gas analysis technique of Van Slyke and Neill (11); hemoglobin per unit volume of blood by Palmer's (12) method; blood cell volume by hematocrit; red corpuscle counts; erythrocyte osmotic resistance by

Hastings' (7) method; and plasma bicarbonate calculated from the Henderson-Hasselbalch equation from the total CO_2 by Van Slyke and Neill (11) method, and from the pH by the method of Hastings and Sendroy (13).

RESULTS. 1. *Blood volume* is markedly decreased in the first week of exercise in three of the four dogs (fig. 1). Two recovered during the next two weeks of continued exercise, while one (dog 3, a swimmer) recovered at the end of four weeks of continued exercise. All dogs, after six weeks of exercise, showed a blood volume higher than normal, which persisted throughout the exercise period and for one to five weeks thereafter.

2. *Total cell volume* is reduced in all dogs during the first week of training (fig. 2). Two running dogs showed recovery to normal in the third week of exercise, while the swimmers required five weeks. During the remainder of the period, the two swimmers and one runner showed marked supra-normal cell volume which persisted for four weeks after cessation of exercise, at which time we discontinued observations.

TABLE 2

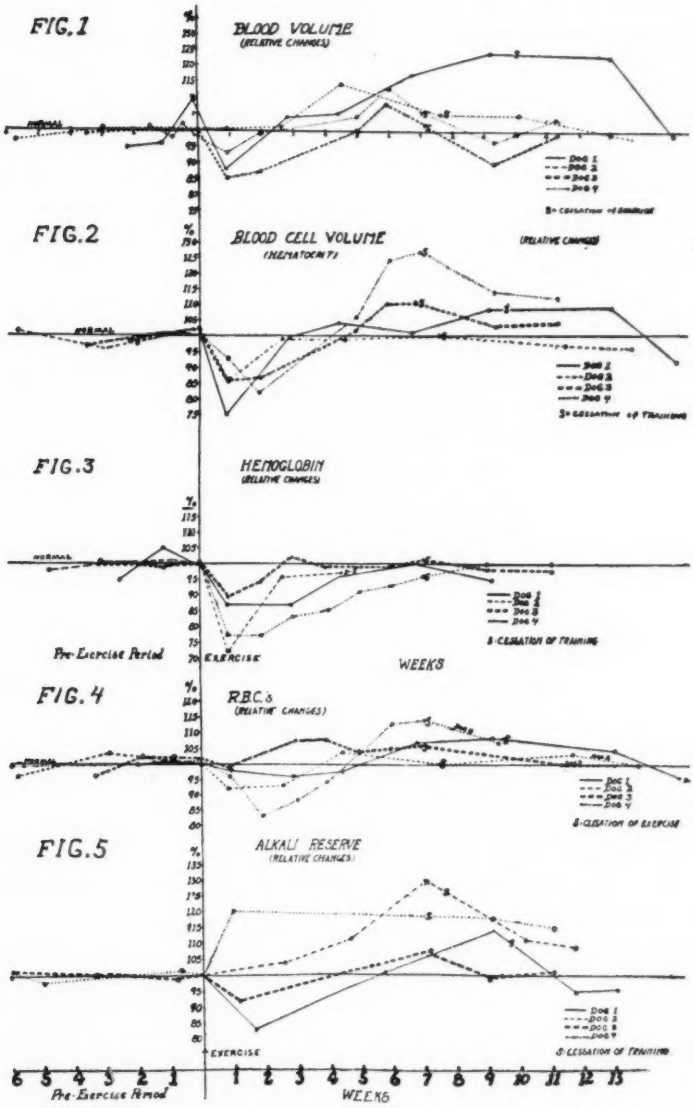
Effect of exercise on the weight of hemoglobin per corpuscle (gm. $\times$ 10^{-12})

	DOG 1	DOG 2	DOG 3	DOG 4
Pre-exercise normal	28.0	29.0	22.6	24.8
After 1 week of exercise	24.6	22.6	20.2	19.9
After 7 weeks of exercise	26.0		21.6	20.8

3. *Erythrocyte number.* All dogs showed a decreased erythrocyte number during the first week of exercise (fig. 4). An erythrocyte count above the normal was observed in each dog after the initial fall, and the polycythemia persisted throughout the exercise period and for four weeks after the cessation of exercise.

4. *Hemoglobin per unit volume of blood* is significantly lowered in all four dogs during the first week's exercise, and recovers irregularly, but in no case exceeds appreciably the normal level (fig. 3). In table 2 the weight of hemoglobin per corpuscle is seen to decrease with exercise. This is in accord with work reported by Thörner.

5. *Total circulating hemoglobin.* The total circulating hemoglobin of the body is increased significantly by five to seven weeks of exercise (table 3). Because of the fact that the polycythemias and high blood volumes in our trained dogs usually persisted for four weeks or longer after cessation of exercise, we are led to believe that the "oxygen debt" of exercise is the stimulus to blood formation (in exercise). Presumably an anoxemia based on anemia would cease to operate as soon as the normal oxygen capacity of the blood has been restored. Some of the members of the



Figs. 1–5. Relative changes in various blood components during a period of physical exercise.

1921 expedition into the Peruvian Andes (Barcroft et al., 15) showed a prompt (2 weeks) return to normal of erythrocyte counts upon descending to lower altitudes. These subjects had shown significant polycythemias at high altitudes and these polycythemias were caused by a condition comparable to anemia with respect to arterial oxygen supply.

6. *Alkali reserve.* In both running and swimming dogs, exercise finally induces a supra-normal alkali reserve, although in two dogs the elevation is preceded by a reduction during the early stages of training (fig. 5). The two dogs showing the initial reduction appeared, before training, to be healthier dogs. Walinski (15) has reported increased alkali reserve in man after physical training, but an increase in alkali reserve in trained dogs has not, to our knowledge, been reported previously. Exercise, it seems, "chemically trains" the blood by increasing its content of available base for the neutralization of acids. It is well known that during exercise CO_2, lactic and other acids are formed in the muscles. Carbon dioxide at all times and lactic acid sometimes appears in the blood during exercise.

TABLE 3

Effect of exercise on total circulating hemoglobin

	DOG 1	DOG 2	DOG 3	DOG 4
	per cent	*per cent*	*per cent*	*per cent*
Pre-exercise normal	100	100	100	100
After 1 week of exercise	76	73	76	72
After 5 weeks of exercise		113		
After 6 weeks of exercise			107	106
After 7 weeks of exercise	124			

Since these substances must be buffered, their very presence in excess in blood may induce the formation of a greater alkali reserve.

7. *Erythrocyte osmotic resistance* increases significantly with training in the two swimming dogs, but decreases slightly in dog 5, a runner (table 1). One of the swimming dogs failed to show an increased cell resistance during the first week of exercise but he also failed to show a significant reduction of hemoglobin during that time (dog 4, fig. 3 and table 1). Dog 5 showed a decreased cell resistance in the first week of exercise, accompanied by a reduction of 15 per cent in hemoglobin per unit volume of blood. It will be noticed that changes in osmotic resistance of corpuscles occur simultaneously with reduction in hemoglobin per unit volume of blood (fig. 3, table 1). (Hemoglobin of dog 5 does not appear in the tables.) Since only our swimming dogs showed increased osmotic resistance of erythrocytes during the exercise period, we suggest that this may have been caused by the slightly reduced (by 2°C.) body temperature, which we observed in them immediately after swimming.

SUMMARY

The following changes in dog's blood due to a period of physical exercise are reported:

1. During the first week of exercise, a reduction of blood volume, cell volume, erythrocyte number, and hemoglobin per unit volume of blood occurred.

2. During the period of exercise, the blood volume, the total cell volume, and the erythrocyte count, not only returned to normal, but exceeded the normal. The hemoglobin per unit volume of blood returned to normal, but did not exceed the normal, although exercise finally induced an increase in the total circulating hemoglobin. A decreased weight of hemoglobin per corpuscle was observed throughout the exercise period.

3. An increase of alkali reserve in both running and swimming dogs occurred.

4. A significant increase of erythrocyte osmotic resistance occurred in two swimming dogs, but a slight decrease of resistance occurred in one running dog. It is suggested that body temperature may be a factor in causing erythrocyte osmotic resistance changes.

5. Since the polycythemia and supra-normal blood volume persisted for about four weeks after the cessation of exercise, it is concluded that the oxygen debt of exercise is the stimulus to the increased blood formation during exercise.

The authors wish to express their appreciation to Dr. A. J. Carlson for valuable advice and criticism rendered during the execution of this work.

REFERENCES

(1) Broun, G. O. J. Exp. Med. **37**: 207, 1923.
(2) Broun, G. O. J. Exp. Med. **37**: 187, 1923.
(3) Rous, P. and O. H. Robertson. J. Exp. Med. **25**: 651, 1917.
(4) McMaster, P. D., G. O. Broun and P. Rous. J. Exp. Med. **37**: 395, 1923.
(5) Thörner, W. Arbeitsphysiol. **2**: 116, 1929.
(6) Thörner, W. Arbeitsphysiol. **5**: 516, 1932.
(7) Hastings, A. B. U. S. P. H. Bull. no. 117: 17–19, 1921.
(8) Steinhaus, A. H. Physiol. Reviews **13**: 119, 1933.
(9) Rice, H. A. and A. H. Steinhaus. This Journal **96**: 529, 1931.
(10) Chang, H. C. and G. A. Harrop. J. Clin. Invest. **5**: 393, 1928.
(11) Van Slyke, D. D. and J. M. Neill. J. Biol. Chem. **61**: 523, 1924.
(12) Palmer, W. W. J. Biol. Chem. **33**: 119, 1918.
(13) Hastings, A. B. and J. Sendroy, Jr. J. Biol. Chem. **61**: 695, 1924.
(14) Walinski. Veroffentl. a. d. geb. d. Heeressanitatswesens **78**: 37, 1925.
(15) Barcroft, J. et al. Phil. Trans. Roy. Soc., London, Ser. B, **211**: 351, 1922.

THE EFFECT OF ERGOTAMINE UPON GLYCOSURIA AND HYPERGLYCEMIA PRODUCED BY STIMULATION OF THE SUPERIOR CERVICAL SYMPATHETIC GANGLION

DAVID A. CLEVELAND

From the Department of Surgery, Northwestern University Medical School, Chicago

Received for publication June 17, 1935

In a previous communication Davis, Cleveland and Ingram presented evidence to show that in well fed cats stimulation of the superior cervical ganglion and of the stellate ganglion resulted in glycosuria and hyperglycemia. No glycosuria and only an insignificant rise in blood sugar resulted from stimulation of the sciatic nerve or the cervical sympathetic trunk. It was also shown that in three cats with properly placed hypothalamic lesions, and in three cats in which the splanchnic nerves had been sectioned as they pierced the diaphragm, stimulation of the superior cervical ganglion did not produce glycosuria or hyperglycemia.

Because of the debated effect of ergotamine, or the closely related ergotoxine, on carbohydrate metabolism we have used it in conjunction with faradic stimulation of the superior cervical ganglion. Sollmann describes ergotoxine as diminishing hyperglycemia, arresting epinephrine and theobromine glycosuria and rendering the kidneys less permeable to sugar. In large doses its action paralyzes and in moderate doses stimulates the sympathetic mechanism.

METHOD. In order to determine the effect of ergotamine on the glycosuria and hyperglycemia resulting from faradic stimulation of the superior cervical ganglion, sixteen experiments were performed on twelve female cats. In each experiment blood was first withdrawn from the femoral vein for a blood sugar determination, and then the cat was anesthetized by an intravenous injection of 13 mgm. of sodium pentobarbital per kilogram of body weight. A urethral catheter was introduced and the bladder completely emptied of urine. Six cats were given subcutaneous injections of 0.5 mgm. of ergotamine tartrate per kilo of body weight. After the superior cervical sympathetic ganglion had been isolated from the rest of the field by a rubber dam it was stimulated with the faradic current. The Harvard inductorium connected to a dry cell battery and with the secondary coil set at 8 cm. was used to stimulate the ganglion. Three stimuli of 30 seconds each were given in each experiment. Urine was

collected before, during, and at 15 minute intervals for 2 hours after stimulation. Blood was collected one hour after the stimulation, because previous experiments had shown that the hyperglycemia was greatest at that time.

Four of the cats showed no sugar in the urine but one developed a moderate and one a heavy glycosuria. The four that did not develop glycosuria also did not show a rise in the blood sugar beyond 35 mgm. per 100 cc. of blood. The cat which developed a moderate glycosuria had a coincidental rise in the blood sugar from 88 to 227 mgm., while the cat with the heavy glycosuria showed a rise in blood sugar from 106 to 258 mgm.

The second series of six cats were tube fed with 80 cc. of whole milk four hours before the experiments were performed. Four of the six were given subcutaneous injections of 1.5 mgm. of ergotamine per kilo of body weight before stimulation. Two animals were used as controls. The four that received ergotamine did not show glycosuria following stimulation and while there was an average blood sugar rise of 59 mgm. in no instance was a blood sugar level reached high enough to pass the kidney threshold. In the two controls glycosuria developed promptly. The blood sugar of one cat rose from 100 to 200 mgm. and the other rose from 100 to 301.

The third of this series of experiments consisted of using two of the cats that had received smaller doses of ergotamine and did not develop glycosuria and two of those that had received larger doses and did not develop glycosuria. These animals were well fed for 2 weeks after the first experiments and then subjected to stimulation of the superior cervical ganglion under sodium pentobarbital anesthesia (13 mgm. per kilo) but received no ergotamine. All four showed a prompt heavy glycosuria and a marked hyperglycemia, averaging between 227 and 348.

From these experiments it can be seen that large doses of ergotamine tartrate prevent the glycosuria and hyperglycemia that normally follow stimulation of the superior cervical ganglion. The pupils of the four cats which received 1.5 mgm. of ergotamine were at first widely dilated and did not react to light, but at the time of stimulation there was sluggish reaction. The pupils of the six cats which had been injected with 0.5 mgm. of the drug were dilated but reacted fairly well to light, but not as actively as the pupils of the control animals. This suggests both a peripheral and a central action of ergotamine. The pupillary reactions of the two cats which developed a definite glycosuria and hyperglycemia following injection of the smaller dose of the drug were no different than the reaction in the cats which had received the same dosage of ergotamine and did not show either glycosuria or hyperglycemia.

SUMMARY

While from these experiments a definite statement as to the exact site in the sympathetic system of the action of ergotamine cannot be made,

it may be concluded that ergotamine inhibits the action of the sympathetic nervous system, that smaller doses usually prevent and larger amounts are more certain to prevent glycosuria and hyperglycemia following stimulation of the superior cervical ganglion in cats which have a sufficiently high glycogen reserve. Further proof is added that stimulation of the superior cervical ganglion by a faradic current will in normal animals result in an immediate glycosuria and rise in the blood sugar.

ACIDOSIS AS A FACTOR OF FATIGUE IN DOGS[1]

F. W. SCHLUTZ, MINERVA MORSE AND A. B. HASTINGS

From the Lasker Foundation for Medical Research and the Department of Pediatrics, University of Chicago, Chicago, Illinois

Received for publication July 11, 1935

Earlier experiments on the effects of fatigue (1) have shown that moderate exercise continued to exhaustion often produces an acidosis in the blood serum of the dog which is characterized by a low bicarbonate and a high lactate concentration. The serum pH under these conditions may fall, but usually it remains within or rises above the normal range as the result of overventilation. The object of the present experiments was to determine whether this acidosis is a primary factor of fatigue in the dog. Our plan consisted in preventing the acidosis of fatigue by the oral administration of sodium bicarbonate and in superimposing upon the acidosis of fatigue an acidosis produced by the administration of ammonium chloride.

Dennig, Talbott, Edwards and Dill (2) and Dennig, Peters and Schneikert (3) have reported a few similar experiments, but with the human subject and with more vigorous exercise, in which they have shown that the ability of the subject to run and the capacity of the blood for neutralizing lactic acid were reduced when the experiment was begun in a state of acidosis. The lactate concentration at exhaustion was higher in the alkalosis experiments. Hartmann and von Muralt (4) also found a greater rise in blood lactate in the alkalosis group than in the acidosis group when the subjects performed a uniform exercise of running up and down a stairs. This effect was especially marked at high altitudes.

EXPERIMENTAL PROCEDURE. The same dogs were used and the same procedure was followed in regard to modes of exercise, taking of samples and methods of analysis as in experiments previously described (1). The dogs were maintained on a uniform diet; their weight remained practically constant. Alkalosis was produced by administering 9 to 12 grams of sodium bicarbonate in 3 to 4 gram doses at half-hour intervals, starting one to one and one-half hour before exercise and acidosis, by a single dose of 3 grams of ammonium chloride given one and one-half hour before exercise. Previous experiments have shown that this provided an alka-

[1] This work has been conducted under a grant from the Douglas Smith Foundation of the University of Chicago.

losis or acidosis which lasted for some hours. Since dog 4 could not tolerate the ammonium chloride by mouth, it was given by rectum. It is of passing interest that this resulted in an acidosis. Control experiments were performed before and after the alkalosis or acidosis experiments to make provision for effects of training. Experiments on a given dog were usually conducted at weekly intervals. In the control experiments dogs 3 and 4 were given water before exercise in the same amounts as in the solutions of ammonium chloride and sodium bicarbonate in order to maintain uniform water conditions in all experiments to be compared. This precaution was not taken in the case of dogs 1 and 2. With the exception of one very long ammonium chloride experiment on dog 3, the dogs were exercised to exhaustion.

TABLE 1

Average values to illustrate the extent of alkalosis or acidosis

CONDITIONS	TIME OF DRAWING SAMPLE	pH				BICARBONATE mM PER LITER			
		Dog 1	Dog 2	Dog 3	Dog 4	Dog 1	Dog 2	Dog 3	Dog 4
After ingestion of $NaHCO_3$	Before exercise	7.58	7.57	7.56		28.1	30.7	37.5	
	At exhaustion	7.64	7.49	7.49		23.3	19.1	23.2	
	After 2 hours' rest	7.49	7.52	7.46		26.7	28.1	24.4	
Control	Before exercise	7.46	7.44	7.48	7.46	22.4	21.0	24.8	20.9
	At exhaustion	7.46	7.36	7.41	7.44	16.3	12.8	18.0	15.1
	After 2 hours' rest	7.43	7.47	7.45	7.46	20.8	21.5	24.0	20.3
After ingestion of NH_4Cl	Before exercise	7.36	7.37	7.36	7.37	16.4	16.4	17.1	17.0
	At exhaustion	7.32	7.33	7.37	7.41	11.5	12.8	15.1	14.6
	After 2 hours' rest	7.33	7.37	7.35	7.30	14.3	16.1	17.6	16.4

The samples of blood serum were analyzed for pH, total CO_2, lactate and sugar. Bicarbonate concentrations were calculated from the results of pH and total CO_2 determinations.

Discussion of results. Using four dogs as subjects we have performed eight experiments in which an alkalosis was produced by giving sodium bicarbonate and eleven in which administration of ammonium chloride caused an acidosis.

The average degree of alkalosis or acidosis and the extent to which it persisted throughout the experiments are shown in table 1. The administration of sodium bicarbonate produced an initial elevation of serum pH and bicarbonate concentration; at exhaustion the bicarbonate concentration and the pH fell in most instances to within normal limits. An exception is to be noted in the case of dog 1, exercised on the treadmill,

whose pH was elevated still farther at exhaustion. The administration of ammonium chloride on the other hand produced an initial depression of pH and bicarbonate concentration, and at exhaustion, values usually below the corresponding exhaustion values in the control experiments. The decrease in bicarbonate concentration from the initial to the exhaustion sample, however, was not so great as in the control experiments. Samples drawn two hours after exhaustion indicated that with few exceptions the effect of the sodium bicarbonate or ammonium chloride still persisted.

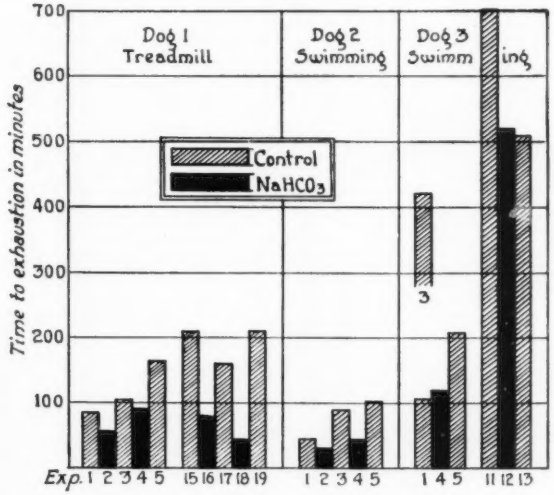


Fig. 1. The effect of an experimentally produced alkalosis on the capacity of the dog for work. The uncompleted column represents a control experiment in which the dog ran an abnormally long time. The completed column below represents the preceding or subsequent control experiment.

Figures 1 and 2 show the length of time the dogs could run before exhaustion in the consecutive experiments of each series. We have taken this time to exhaustion as a measure of their capacity to perform work. Since the capacity for performing work increased with training and since a progressive decrease in cell volume due to blood loss in drawing samples occurred, it has seemed preferable to base conclusions upon a comparison of the results of individual experiments with those of the corresponding control experiments, rather than upon averages of each series.

The results for the first two dogs studied, 1 and 2, are striking. In every case the capacity for work was reduced when exercise was begun in a state of alkalosis. At exhaustion, in these experiments, the pH and

bicarbonate concentrations of the serum were practically normal, but the concentrations of serum lactate were usually higher than in the control experiments (fig. 3). When exercise was begun with the dog in a state of acidosis the opposite result was obtained; in four out of six experiments the dogs were capable of exercising longer than in their control experiments. The pH and bicarbonate concentrations of the serum were usually lower throughout the experiment, but the serum lactate concentrations tended to be less than in the control experiments.

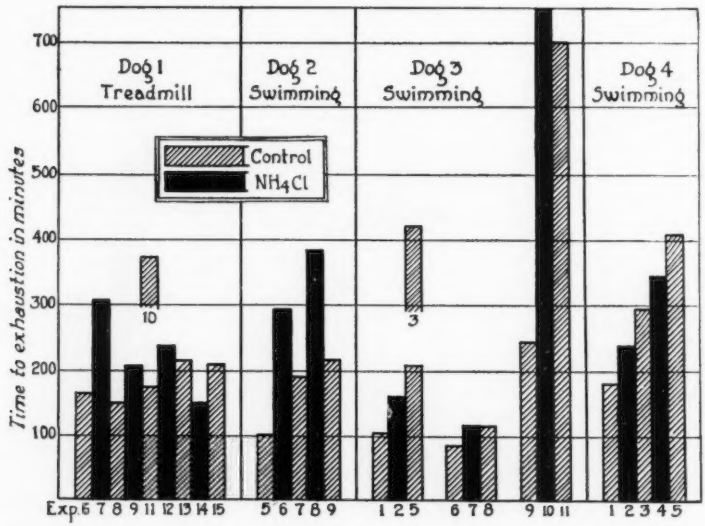


Fig. 2. The effect of an experimentally produced acidosis on the capacity of the dog for work. The uncompleted column represents a control experiment in which the dog ran an abnormally long time. The completed column below represents the preceding or subsequent control experiment.

These results were not reproduced in similar experiments with dogs 3 and 4. While the alkalosis or acidosis experiment showed the same relation to the control experiment just preceding that was shown in the case of dogs 1 and 2, this relation did not hold when they were compared with the control experiment following except in one instance, an alkalosis experiment. In spite of the fact that an effort was made in these later experiments to eliminate the training factor by exercising the dogs for several weeks before the experiment was begun, the control experiments were marked by an irregularity of response which has masked any small effects of the ammonium chloride or sodium bicarbonate. The results in the case of dog 4 were typical of training. The ammonium chloride

had no apparent effect. There was no evidence in any of these experiments, however, that an ammonium chloride acidosis superimposed upon the acidosis of exercise had hastened the onset of fatigue, or that the prevention of an acidosis by administration of sodium bicarbonate had postponed it.

It is necessary to consider how other factors than fatigue or a state of alkalosis or acidosis may have influenced the results. Training may have masked small beneficial effects of ammonium chloride in the case of

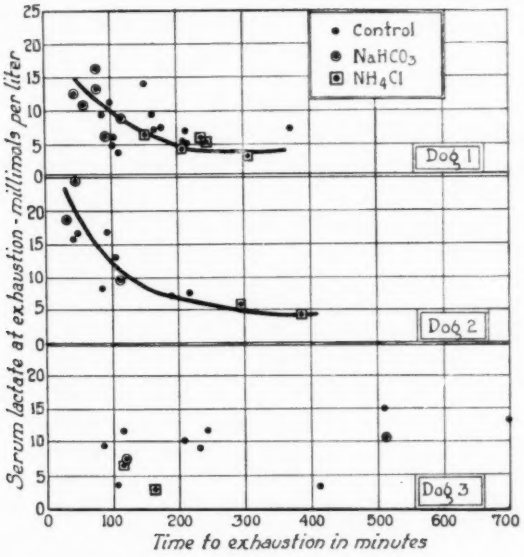


Fig. 3. The relation between the concentration of serum lactate at exhaustion and the capacity of the dog for work.

dog 3, but could not otherwise have affected the relative results in either the sodium bicarbonate or ammonium chloride experiments.

It was impossible to maintain a rigid uniformity of conditions. In the swimming experiments the temperature of the water was kept at 38°C., but the intensity of exercise could not be controlled. In experiments on the motor-driven treadmill the speed was constant but the room temperature varied from 20.0° to 28.5°C. Most of the experiments, however, were performed with a room temperature of 24 to 26°C. With very few exceptions, differences in room temperature could not have been the factor which determined the relative lengths of the sodium bicarbonate, ammonium chloride or control experiments on the treadmill. A low room temperature (20°C.) may have been the cause of the one unusually long

control experiment. The similarity of results with exercise on the treadmill and by swimming would seem to show that, at least for dogs 1 and 2, uncontrollable differences in conditions were not factors which determined the relative results.

A low serum sugar concentration occurred at exhaustion in a few experiments with dog 2, but the data show that the same relation would have existed between the time to exhaustion in the ammonium chloride or sodium bicarbonate experiments and that in the controls, even if lack of fuel had been considered the determining factor in the onset of fatigue.

In these experiments rectal temperature at exhaustion varied from 38° to 42.5°C. It was usually higher in treadmill than in swimming experiments. The experimental alkalosis or acidosis had no apparent influence on the rectal temperature at exhaustion.

The possibility of water metabolism as a factor determining the results must be considered. While dogs 3 and 4 were given the same amount of water before exercise in the control experiments as was contained in the sodium bicarbonate and ammonium chloride solutions, dogs 1 and 2 were not given water in the control experiments. However, the similarity of results for dogs 1 and 2, exercised on the treadmill and by swimming respectively, makes it appear that differences in water intake were not a determining factor. Experiments have shown that the dogs take in large quantities of water while swimming.

Figure 3 shows the results of an attempt to correlate serum lactate at exhaustion with the length of time the dogs were capable of exercising. In the case of dogs 1 and 2 an inverse relationship appears to exist. The lines are drawn free hand through the points. There is obviously considerable scattering of the points, but they seem to indicate that regardless of whether the animal is normal, has had sodium bicarbonate or ammonium chloride, the higher the serum lactate, the shorter the time it is capable of working. Such a relationship did not exist in the case of dog 3. This dog was able to swim for long periods and showed little rise in serum lactate concentration until exhaustion. At exhaustion there was a sudden, marked rise which bore no relation to the length of the exercise period. Data on dog 4 were insufficient to show whether or not there was a relation between serum lactate and capacity to exercise.

SUMMARY

The effect of experimentally produced alkalosis and acidosis on the capacity of dogs for work has been studied.

Alkalosis induced by the administration of sodium bicarbonate often reduced the capacity of dogs for muscular exercise although the acid-base balance of the blood was normal at exhaustion. Conversely, acidosis

following ingestion of ammonium chloride often exerted a favorable effect and in no case appreciably reduced the capacity of the dogs for exercise.

Ingestion of sodium bicarbonate promoted the formation of lactic acid in the exercising dog while that of ammonium chloride depressed its formation.

Since the onset of exhaustion often occurred earlier when the acidosis of exercise was compensated by giving sodium bicarbonate, and since it was at least not hastened by superimposing an ammonium chloride acidosis on the acidosis of exercise, it would appear that the acidosis accompanying physical exercise is not to be regarded as a causal factor of fatigue in dogs.

The authors are deeply indebted to Dr. Grover Hulla and Miss Helen Oldham for valuable assistance in the experimental work.

REFERENCES

(1) Schlutz, F. W., A. B. Hastings and M. Morse. This Journal **111**: 622, 1935.
(2) Dennig, H., J. H. Talbott, H. T. Edwards and D. B. Dill. J. Clin. Invest. **9**: 601, 1931.
(3) Dennig, H., K. Peters and O. Schneikert. Arch. Exp. Path. u. Pharmakol. **165**: 161, 1932.
(4) Hartmann, H. and A. v. Muralt. Biochem. Ztschr. **271**: 74, 1934.

THE EXCRETION OF PHENOL RED BY THE DOG[1]

JAMES A. SHANNON

From The Department of Physiology, New York University College of Medicine, New York City

Received for publication June 20, 1935

Evidence that phenol red is excreted in part by tubular secretion in the dog's kidney has been presented by Marshall and Vickers (1923), Marshall and Crane (1924) and Marshall (1931). Briefly, this evidence is as follows: 1. Under normal conditions a considerable fraction of the dye in the plasma is combined with the plasma proteins, and since an abundance of evidence indicates that the glomerular filtrate is normally protein-free, this fraction is rendered unavailable for filtration, leaving an insufficient quantity of filtrable dye to account for its excretion when reasonable assumptions are made concerning the rate of blood flow through the kidneys. 2. The phenol red clearance at plasma levels of 0.21 to 0.54 mgm. per cent is from 1.40 to 2.64 times the simultaneous creatinine clearance in the normal dog, despite the fact that only 25 per cent of the dye is present in a filtrable form. 3. In anesthetized dogs the clearance is not independent of plasma concentration, but decreases as the latter is raised. 4. When administered intravenously, phenol red is stored in the convoluted tubules of the kidney of animals rendered anuric by section of the cord. 5. In dogs under paraldehyde anesthesia the extraction ratio of the dye (venous/arterial blood) at low concentrations in the plasma is larger than the ratio: filtrable/total dye.

It must be recognized that, although the combination between phenol red and plasma protein is a reversible one (Grollman, 1926), filtration, as this term is applied to the glomerulus, is unable to extract bound dye from the plasma. No change occurs in the concentration of free dye in the unfiltered plasma in consequence of filtration to alter the equilibrium between the free and bound fractions, and thus effect the liberation of the latter. The first three lines of evidence given above are acceptable as indicating tubular secretion as a major mode of excretion. The phenomenon of storage in the convoluted tubules (4) and the large magnitude of the A/V extraction ratio (5) are, however, open to the criticism that tubular absorption may occur without excretion into the lumen of the

[1] A preliminary report of this work is contained in Proc. Soc. Exp. Biol. and Med. **32**: 977, 1935.

tubule (as demonstrated by Chambers, 1935, in *in vitro* cultures of the mesonephric tubules of the chick); considered alone, therefore, these arguments are inconclusive, though perhaps they may be taken as supplementing the other evidence.

Except for a few simultaneous phenol red and creatinine clearances at very low phenol red plasma concentration (Marshall, 1931) there are no data that will permit the comparison of the rate of excretion of the dye with the rate of excretion of other urinary constituents. The two experiments of Marshall and Crane (1924) on the influence of plasma concentration on rate of excretion were obtained on anesthetized and operated animals and leave the phenomenon of the curvilinear relationship without satisfactory definition. The experiments described below were performed with special reference to these two points. Inulin clearances were used as a standard of reference since the available information indicates that the clearance of this substance is close to, if not identical with, the rate of glomerular filtration.

CHEMICAL METHODS. Coagulation of the blood was delayed by the use of colorless heparin (Connaught Laboratories), which permits the direct colorimetric determination of total phenol red in plasma. After removal of the plasma for the sugar determinations, sufficient oxalate was added to permanently prevent coagulation in the plasma that was to be dialysed. Inulin and glucose were determined as described by Shannon and Smith (1935) by the Shaffer-Somogyi method, on an iron filtrate. While the presence of phenol red obscured the iodine color at the point where starch is added in the thiosulphate titration, it did not interfere with the starch-iodine endpoint itself. Phenol red was determined directly in the plasma by colorimetric comparisons with standards containing 0.1 to 1.0 mgm. per cent. One-tenth cubic centimeter of saturated Na_2CO_3 was added to each 3 cc. of plasma, diluted urine and standard to develop the full color of the dye. When an $\epsilon 74$ Wratten filter is used in the Duboscq colorimeter with micro cups and prisms, a blank (equal to 0.04–0.10 mgm. per cent of phenol red) is obtained on a control sample of serum. This blank is perfectly additive in respect to the addition of known quantities of phenol red, and consequently the dye can be recovered with an error not exceeding 2 per cent by direct colorimetric readings. In each experiment the blank was determined on a sample of plasma drawn prior to the injection of phenol red, and applied to all phenol red determinations made during the course of that experiment. Plasmas containing more than 1.0 mgm. per cent of the dye were diluted to about this value with 0.9 per cent saline before colorimetric analysis. The clarity of the serum was maintained at a maximum by keeping the animal on a fat-free, high carbohydrate diet for 48 hours preceding the experiment.

Free or filtrable phenol red was determined by filtration-dialysis at 37°C., p CO_2 = 38 mm., thus effecting a separation of intermicellar fluid from plasma in equilibrium with the bound dye, with no dilution of the constituents in the system. Blood was drawn and centrifuged under oil, or drawn in a syringe containing dry heparin, and immediately centrifuged in a full tube, capped to prevent aeration, to insure readjustment of the plasma to the original pH when re-equilibriated with CO_2. Duplicate determinations yield figures for free dye at any one total concentration agreeing within 2 per cent.

Grollman (1925) found that at constant pH and at room temperature, the binding of phenol red by plasma protein obeys the adsorption isotherm; $x/m = Kc^{1/n}$ where x/m equals the millimols of dye adsorbed on m grams of adsorbent and c is the equilibrium concentration in mM. per liter; k and 1/n are constants. For pig serum he found that the constant 1/n approximated unity and the equation allowed reduction to $x/m = Kc$, indicating that the percentage of dye bound at all concentrations in the plasma was the same. Our observations on dog plasma do not allow this reduction. As can be seen from the data in figure 1, obtained on two samples of dog serum, when the concetration of total dye in the plasma is varied from 1 to 65 mgm.

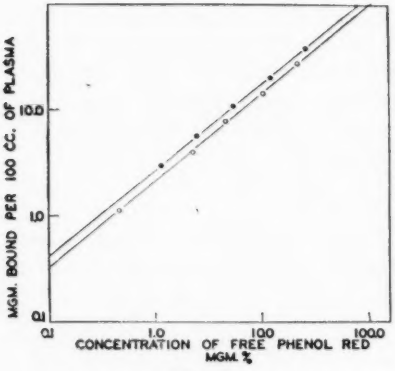


Fig. 1

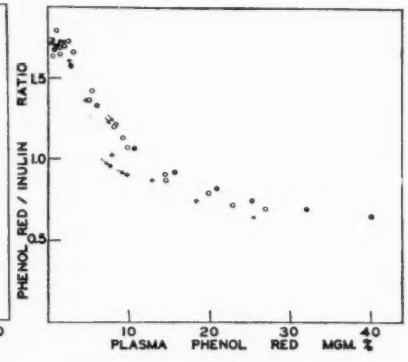


Fig. 2

Fig. 1. Chart showing the relation between the milligrams phenol red bound per 100 cc. plasma and the equilibrium concentration in milligrams per 100 cc. water. Note the slope of the line on this logarithmic plot is not 1.0 but 0.83, indicating that the percentage of filtrable dye increases with increasing total concentration in the plasma.

Fig. 2. Chart showing the variation in the phenol red/inulin clearance ratio at various plasma levels of phenol red. It should be noted that the variation in the value of this ratio is due to absolute changes in the phenol red clearance, the inulin clearance remaining relatively constant. (Ninety per cent of the inulin clearances in the four experiments given here were within 60 to 73.6 cc. per minute. Also see table 1.) These values were obtained upon three dogs, each one indicated by a different symbol. The points indicated by arrows were obtained upon a stationary or ascending plasma concentration of dye.

per cent, the constant 1/n has a value approximating 0.82 to 0.85. This fact leads to an increasing fraction of free phenol red at increasing plasma concentration.

Ultra filtrates were not made on all samples of blood in the experiments reported here, due to the relatively large quantities of blood necessary; the free phenol red was determined mainly at the higher concentrations, and at lower concentrations the free fraction was calculated from curves having the same slope as those in figure 1, and passing through the points determined experimentally. Such extrapolations involve only the assumption that subsequent bleeding in any one experiment does not significantly alter the binding power of the plasma.

EXPERIMENTAL. Trained female dogs weighing from 14 to 21 kilos were used throughout. Inulin in doses of 2.0 to 3.0 grams per kilo, dissolved at 85°C. as a 20 per cent solution in 0.6 per cent saline, was given by intravenous infusion. In the long experiments two infusions of inulin were given to maintain the plasma concentration of the substance at a high level. The rate of urine flow was usually maintained above 1 cc. per minute by the administration of water, or 1 per cent saline, at intervals of 2 hours. Phenol red was given intravenously in most cases as a 10 per cent solution of the sodium salt in doses from 200 mgm. to 4 grams; in a few experiments the dye was given subcutaneously. Phlorizin was given in doses of from 300 to 400 mgm. per kilo, one half intravenously and the remainder subcutaneously. Otherwise all methods were identical with those previously reported from the laboratory.

RESULTS AND DISCUSSION. The most interesting fact about the secretion of phenol red is the curvilinear relationship displayed between the clearance of this dye and its concentration in the plasma, as roughly indicated by previous studies. This relationship affords an opportunity to examine some of the factors involved in the process of tubular secretion.

In figure 2 we have presented data obtained by a comparison of simultaneous phenol red and inulin clearances from four experiments on three dogs. These data reveal the precise nature of this curvilinear relationship. As the phenol red concentration in the plasma is increased, the absolute value of the phenol red clearance, and therefore of the phenol red/inulin clearance ratio, decreases in a systematic manner. It is desirable to consider the phenol red/inulin clearance (PR/I) ratio, rather than the absolute phenol red clearance, in order to permit different dogs with varying absolute clearances to be compared.

The inulin clearance is known to be independent of the plasma inulin concentration (Shannon, 1935; see also table 1) and it is quite constant in any one series of observations if the dog is handled carefully. It is shown here (see table 1) that the inulin clearance is not affected by the administration of large doses of phenol red. At low plasma levels the PR/I ratio averages 1.73; as the plasma phenol red level is raised, this ratio falls until at plasma levels above 25 mgm. per cent it is less than 0.7. In a fifth series of observations not included in figure 2 the PR/I ratio was followed through eleven periods in which the plasma level of the dye fell from 67 to 10 mgm. per cent. This series was omitted from the figure because no data were obtained on the maximum value of the PR/I ratio. Between 40 and 10 mgm. per cent, however, the PR/I ratios of this experiment follow closely those observed in the other experiments. In a sixth experiment, consisting of eight periods with plasma levels varying from 40 mgm. per cent to 1.5 mgm. per cent, the PR/I ratio had a maximum value of 2.06 at the lower plasma levels, a figure significantly

higher than was observed in the other experiments, and the curve generated at higher plasma levels was similar to the data in figure 2, but consistently higher at all plasma levels, by 10 to 30 per cent.

The maximum PR/I ratio in all the animals examined has been close to 1.7, but in the different experiments even with the same dog, this ratio varies somewhat, and in one instance was as high as 2.1. In all instances

TABLE 1

Experiment on normal dog showing the depression of the clearance of phenol red and the phenol red/inulin ratio as the plasma level of phenol red is raised

Note that the same value is obtained on both "rising" and "falling" concentration of phenol red in the plasma.

PERIOD	DURATION OF PERIOD	URINE FLOW	PLASMA LEVEL			CLEARANCE		RATIO OF CLEARANCES	PHENOL RED EXCRETED		
			Inulin	Phenol red, total	Phenol* red filtrable	Inulin	Phenol red	$\frac{\text{Phenol red}}{\text{Inulin}}$	Total	By filtration	By secretion
	min.	*cc. per min.*	*mgm. per cent*	*mgm. per cent*		*cc. per min.*	*cc. per min.*		*mgm. per min.*	*mgm. per min.*	*mgm. per min.*
1	22	2.09	308	1.48		66.2	109	1.65			
2	22	1.68	192	1.09		71.7	129.5	1.80			
Phenol red 80 mgm. per kilo subcutaneously											
3	21	2.43	88.5	8.07		69.0	83.8	1.20			
4	23	3.47	65.5	8.20		66.4	80.4	1.21			
Phenol red 120 mgm. per kilo by vein											
5	31	2.48	380	22.8	9.30	69.2	50.2	0.71	11.45	6.43	5.02
6	33	1.30	200	14.35	5.51	71.4	64.3	0.90	9.23	3.93	5.40
7	31	0.645	127	9.19	3.35	65.5	74.5	1.13	6.84	2.19	4.65
8	30	1.17	87.5	5.55	1.92	73.6	105.0	1.42	5.83	1.41	4.42
9	30	2.62	55.2	3.13	1.02	69.6	114.0	1.66	3.57	0.71	2.86
10	34	1.27	37.0	2.10		68.6	116.5	1.70			
11	33	1.06	24.5	1.30		68.8	116.0	1.69			

* Bloods 5 and 6 were determined by ultra filtration; bloods 7 to 9 were read off a curve as described under methods.

this ratio approximates a constant at levels of the dye below 1.5 mgm. per cent; if it continues to increase as the concentration of phenol red falls below this point, the change is so small that present technique does not reveal it. At just what plasma level a constant PR/I ratio is reached has not been determined; it is probably never higher than 3.0 mgm. per cent in any animal and is certainly lower in most.

In most instances where the phenol red concentration exceeded 20 mgm. per cent we have determined the percentage of filtrable dye. The maximum variation in this figure has been from 35 to 44 per cent in the range from 20 to 40 mgm. per cent of total dye.

In a previous series of observations (55 periods on 3 dogs) we have made comparisons of the simultaneous phenol red and xylose clearances. The PR/X ratios, if corrected by multiplying by 0.734 (the average xylose/inulin ratio found by Shannon in the dog (1935)), superimpose themselves within the limits of the data as given in figure 2.

The data given in table 1 and the experiments included in figure 2 show that the depression of the phenol red clearance, which is effected by raising the plasma level of the dye, is truly reversible, the same absolute clearance (or the same PR/I ratio) being obtained at low plasma levels of dye either after a small dose, or when the low level is arrived at by excretion from a high level. The depression of the phenol red clearance is equally reversible at intermediate levels, the same values being reached when the plasma concentration falls from high to an intermediate level, as when it has been held constant at the intermediate level by subcutaneous injection.

Marshall and Crane (1924) have suggested that the curvilinear relationship between plasma level and excretion issues from the fact that tubular storage precedes secretion, and that at a certain concentration the tubular cells become saturated, so that any further increase in the rate of excretion can be brought about only by increasing the free dye in the glomerular filtrate. This view of the mechanism involved can be neither accepted nor denied on the available evidence, but a more exact appraisal of the situation can now be made. One can calculate, from the simultaneous inulin and phenol red clearances, and a knowledge of the concentration of free dye in the plasma, the actual quantity of phenol red excreted by filtration and the quantity excreted by the tubular secretion. Both these moieties can be expressed conveniently in terms of glomerular filtrate, in order to eliminate variations in blood flow to the kidney, as well as errors due to timing and collection of urine samples such as affect the absolute values of the clearances rather than their ratios.

Data expressed in this manner are given for one experiment in table 2. In figure 3, data from another experiment are expressed graphically. (It should be noted that in the latter experiment the inulin clearance was relatively constant throughout, varying only from 65.0 to 71.5.) The solid dots show the actual milligrams of dye, corrected to 100 cc. of concurrent filtrate as suggested above, excreted at various plasma levels. The circles show the corresponding quantity of secreted dye, calculated as the difference between the filtered dye (inulin clearance $\times$ concentration of free dye per cubic centimeter) and the total dye excreted. On analysis, the curve describing total excretion is seen to be composed of

two moieties, a secreted moiety, A, which rises to a constant value at about 5 mgm. per cent of free phenol red, and a filtered moiety, B, which increases in direct proportion (as it must in theory) to the free phenol

TABLE 2

Phlorizin experiment showing the absence of a significant effect upon the phenol red/inulin ratio of a large dose of phlorizin (150 mgm. per kilo intravenously; 150 mgm. per kilo subcutaneously)

PERIOD	DURATION OF PERIOD	URINE FLOW	PLASMA LEVEL			CLEARANCE			RATIO OF CLEARANCES	
			Inulin	Phenol red	Glucose	Inulin	Phenol red	Glucose	Phenol red / Inulin	Glucose / Inulin
	min.	*cc. per min.*	*mgm. per cent*	*mgm. per min.*	*mgm. per cent*	*cc. per min.*	*cc. per min.*	*cc. per min.*		
1	16	2.62	324.0	1.82		79.6	123.3		1.55	
2	17	1.82	223.0	1.20		79.0	126.9		1.60	
Phlorizin and phenol red administered										
3	20	5.1	110	2.82	94.8	67.3	78.0	64.9	1.16	0.97
4	26	4.93	84.6	1.72	91.8	72.0	103.0	70.5	1.43	0.98
5	20	4.75	63.0	1.06	88.0	73.2	113.5	72.2	1.55	0.99

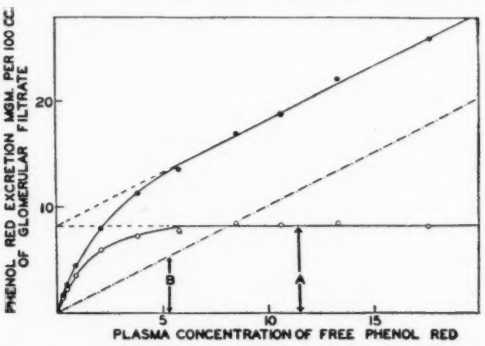


Fig. 3. Chart showing the relation between total, filtered and secreted phenol red and the plasma concentration of the free dye. The solid circles indicate the total phenol red excreted; the dot-dash line, *B*, represents the filtered phenol red (obtained by multiplying the concentration of free dye in the plasma by the inulin clearance, adjusted to 100 cc. per minute); and the open circles indicate the quantity of dye secreted, as calculated by subtracting the filtered moiety from the total. Note that the secreted moiety, *A*, increases with increasing plasma concentration until a constant rate of secretion is reached (5 mgm. per cent free dye).

red. In this experiment the dye excreted by secretion rose from a value of 32 per cent of the total at the highest plasma level to 82 per cent of the total at the lowest plasma level.

This description of the relation between secretion and plasma level cannot be unconditionally accepted, however. Although six other experiments show in general the same relationship as is revealed by the experiment given in figure 3, an eighth experiment shows a secreted moiety (on a falling blood curve) rising from a value of 9.2 mgm. at 40 mgm. per cent to 13.1 mgm. at 15.2 mgm. per cent, and thereafter falling as in figure 3. Moreover, in many experiments, the first period after the intravenous injection of a large dose of dye shows an aberrantly low value for the secreted moiety. These facts suggest that this moiety may be toxically depressed by high concentration, or actually pass through a maximum rather than remain constant, with descending plasma levels.

It should be noted that, if a process of storage in the tubule cells precedes secretion, this storage is not the conditioning factor determining the rate of secretion at low or intermediate plasma levels, since the reversibility of the curve in figure 3 requires equilibrium between the secretory cells and the concurrent plasma level. Whether one may properly apply the word "storage" to such an equilibrium, or whether saturation, in the sense of storage, is the factor that leads to constancy in the secreted moiety at plasma concentrations of free phenol red above 5 mgm. per cent cannot be decided from the type of experiment reported here.

Although our experiments were not designed to study the effect of urine flow on the excretion of phenol red, it is to be noted that the PR/I ratio at a given plasma level of dye remains constant at urine flows from 2.6 to 0.3 cc. per minute. Moreover, the administration of one per cent NaCl solution by mouth does not affect this ratio in comparison to that obtained with water diuresis. The independence of rate of urine flow and phenol red excretion has previously been noted by Marshall and Kolls (1919) on anesthetized dogs.

In two experiments phlorizin in doses of 300 to 400 mgm. per kilo had no influence upon the PR/I ratio. One such experiment is given in table 2. It should be noted in confirmation of Shannon (1935) that, following phlorizin in adequate doses, the glucose clearance rises to the level of the inulin clearance.

SUMMARY

1. The excretion of phenol red in the dog has been examined with special reference to the effect of plasma level. In comparison with simultaneously determined inulin clearances it has been found that at plasma phenol red levels below 1.5 mgm. per cent the phenol red clearance has a maximum value averaging 1.7 times that of the inulin clearance. As the plasma level is raised the phenol red clearance falls, both absolutely and relative to the inulin clearance, until at plasma levels above 25 mgm. per cent the PR/I clearance ratio is less than 0.7.

2. The depression of the phenol red clearance effected by raising the plasma level of the dye is perfectly reversible.

3. The curvilinear relation described in (1) is analysed in terms of the relative fraction of dye that is excreted by filtration and by secretion. At plasma concentration of 0.5 to 1.5 mgm. per cent the relative fraction excreted by secretion approximates 83 per cent, while at plasma concentration of 40 mgm. per cent, only 35 per cent is excreted by secretion.

4. The secretion of phenol red is not specifically influenced by phlorizin.

5. Wide variations in urine flow appear to have no effect upon the rate of excretion of this dye.

REFERENCES

CHAMBERS, R. Proc. Soc. Exp. Biol. and Med. **32:** 1199, 1935.
GROLLMAN, A. J. Biol. Chem. **64:** 141, 1925.
J. Gen. Physiol. **9:** 813, 1926.
MARSHALL, E. K., JR. This Journal **99:** 77, 1931.
MARSHALL, E. K., JR. AND M. CRANE. This Journal **60:** 465, 1924.
MARSHALL, E. K., JR. AND A. C. KOLLS. This Journal **49:** 302, 1919.
MARSHALL, E. K., JR. AND J. L. VICKERS. Bull. Johns Hopkins Hospital **34:** 1, 1923.
SHANNON, J. A. This Journal **112:** 405, 1935.
SHANNON, J. A. AND H. W. SMITH. J. Clin. Invest. **14:** 393, 1935.

THE CLEARANCE, EXTRACTION PERCENTAGE AND ESTIMATED FILTRATION OF SODIUM FERROCYANIDE IN THE MAMMALIAN KIDNEY. COMPARISON WITH INULIN, CREATININE AND UREA

DONALD D. VAN SLYKE, ALMA HILLER AND BENJAMIN F. MILLER[1]

From the Hospital of the Rockefeller Institute for Medical Research, New York, N. Y.

Received for publication July 17, 1935

The present paper is a contribution to attempts to measure the glomerular filtrate, towards which attention was directed by Cushny's filtration-reabsorption theory of urine formation (1926a). If the filtration-reabsorption theory can be proven true, and the true glomerular filtrate can be measured, it will become possible to describee the processes of urine formation more fully because: 1. Any substance whose plasma clearance exceeds the true filtrate must be secreted at least in part by the tubules. 2. Any substance whose clearance over all ranges of plasma concentration equals the true filtrate is in all likelihood excreted by pure filtration.[2] 3. Any substance which shows a plasma clearance below the known glomerular filtrate, and which can be proved to be excreted through the glomerulus alone, presumably undergoes tubular reabsorption, the extent of which may be estimated from the difference between filtrate and clearance.[3]

Rehberg (1926) proposed the creatinine clearance in man as a probable

[1] National Research Council Fellow in Medicine.

[2] The term "clearance" is used in the sense defined by Møller, McIntosh, and Van Slyke (1928), as the volume of blood (or of plasma if plasma clearance is considered) containing the amount of excretory substance that is eliminated per minute in the urine. Strictly speaking, for a substance under category (2), the glomerular filtrate should equal, not exactly the plasma volume indicated by the clearance value, but about 0.95 as much, or the volume of water in the amount of plasma represented by the clearance, the other 0.05 of the plasma being occupied chiefly by non-filterable colloids. This estimate is based on the assumption that the water and filterable solutes of the plasma pass into the filtrate in the same proportions in which they exist in the plasma, an assumption which may be accepted as practically proved by Richards and his colleagues.

[3] The processes of filtration and reabsorption, as they are assumed to occur in accordance with the theory, are outlined more completely in a previous communication (Van Slyke, Rhoads, Hiller and Alving (1934a, p. 363)) together with the work of Richards, Smith, Marshall and their colleagues providing the experimental basis for the theory.

measure of the glomerular filtrate, because "ingested creatinine is concentrated by the kidney to a far greater extent than any other hitherto observed substance in the urine." Other than this presumption, evidence for the identity of creatinine clearance and glomerular filtrate was lacking, however, and it was challenged by Jolliffe and Chasis (1933), and Chasis, Jolliffe, and Smith (1933) and Shannon (1935a).

Because carbohydrates cannot be excreted by the tubules of the aglomerular fish (Marshall and Grafflin, 1928), it seemed probable to Jolliffe, Shannon and Smith (1932) that non-metabolized sugars could be used to measure glomerular filtration. For this measurement they proposed the xylose clearance, which in the dog (Shannon, Jolliffe and Smith, 1932) they found to be about 70 per cent of the creatinine clearance. They believed, therefore, that about 15 to 40 per cent of the creatinine in a dog's urine was excreted by the tubules, and that the xylose clearance represented the glomerular filtrate.

Later, however, it was found in dogfish (Shannon, 1934), in dogs (Richards, Westfall and Bott, 1934; Shannon, 1935b), and in men (Shannon and Smith, 1935) that the xylose clearance was markedly lower than the inulin clearance, which is now believed to represent more nearly the true volume of the glomerular filtrate.

That the larger inulin clearance, more probably than the xylose clearance, approximates the glomerular filtrate was deduced from facts which indicated that inulin is filtered through the glomerular tuft and passes into the urine probably without being, to a significant extent, either reabsorbed from the tubules, or joined there by additional inulin excreted by the tubules. That inulin is filtered in the glomerulus was shown by Richards and his collaborators, who found it in the glomerular filtrate of the frog in the same concentration as in the plasma; it appears probable that similar filtration occurs in the dog. That inulin is probably not reabsorbed by passive diffusion in the tubules was deduced from its high molecular weight, 900 to 5000 (although from this one could not exclude the possibility of active reabsorption). That inulin does not pass in the opposite direction, from blood to tubular lumina, by excretion, appeared improbable from the fact that inulin was found not to be excreted by aglomerular fish (Richards, Westfall and Bott, 1934).

The plasma inulin clearance was found by Richards, Westfall and Bott (1934) and by Shannon (1935b) to be of the same order of magnitude as the creatinine clearance in the dog. This agreement shifted the probability strongly back towards Rehberg's assumption, that the plasma creatinine clearance approximates the glomerular filtrate, at least in the dog. However, the interpretation rested on the assumed non-passage of inulin through the tubular walls, either from blood to lumen or *vice versa*, and the assumption was based on analogy with the aglomerular fish, and on

probable non-diffusibility through the walls; direct evidence of the non-passage was lacking.[4]

Recent histological studies by Gersh do offer such evidence with regard to the excretion of ferrocyanide. Before the development of a special technique by Gersh (1932) it had been impossible to ascertain the location of ferrocyanide accurately by histochemical methods, because the highly soluble salt would diffuse through the tissues before fixation had been completed. Gersh's technique consists in dehydrating a frozen slice of tissue under high vacuum at minus 20°C. At this temperature no liquid phase exists, so that diffusion of soluble salts is prevented and the normal architecture of the tissue is preserved. The dried tissue is then stained with anhydrous reagents.

With this improved method Gersh and Stieglitz (1934) have demonstrated that when the rabbit's kidney excretes much sodium ferrocyanide this substance is found in demonstrable concentration in the filtrate of the glomerular capsule, and in high concentration in the tubular lumen, but is absent from the tubular cells. These results seem to constitute direct evidence that ferrocyanide is excreted entirely by glomerular filtration, without passage through the tubular epithelium in either direction. Gersh and Stieglitz also found that when renal filtration is stopped by section of the spinal cord no ferrocyanide appears in the glomerular capsule or tubular lumen.

These observations of Gersh and Stieglitz suggested to us that ferrocyanide might serve for the measurement of the true glomerular filtrate. It may be used because it is non-toxic in relatively large dosage and can be determined with considerable accuracy in both plasma and urine. We have carried out most of our experiments on a group of dogs with explanted kidneys, since we wished to obtain extraction percentages as well as renal clearances. Doctor Gersh was kind enough to repeat his observations on dogs under the conditions of our experiments, and found the distribution of ferrocyanide in the renal elements to be exactly the same as in the rabbit. He has also found similar ferrocyanide distribution in the renal elements of monkeys and other mammals, as well as in the fetuses of the chick (Gersh, personal communication, 1935).

We have obtained a few clearances with rabbits, but have conducted

[4] It is a pleasure to acknowledge that the present work was begun as the result of A. N. Richards' direct inspiration. Richards recognized the desirability of more evidence concerning the behavior of inulin in the kidney, and suggested to the authors the interest that would attach to measurements of the percentage inulin extraction by the technique of Van Slyke, Rhoads, Hiller and Alving (1934a). Richards' suggestion caused us to postpone work on another phase of renal physiology, and to take up the behavior of inulin. The desirability for data from an additional substance, for which there was direct evidence of tubular non-secretion and non-absorption, led us later to include ferrocyanide in our experiments.

most of our experiments on dogs with explanted kidneys; and have compared the clearances and extraction percentages of sodium ferrocyanide with those of creatinine, inulin and urea.

EXPERIMENTAL. Experiments were performed on dogs in which one kidney had been explanted by the technique of Rhoads (1931, 1934) and the other kidney had been removed, so that the analyses of urine and renal venous blood refer to the same kidney. The operated dogs were the same ones prepared by Doctor Rhoads and used in previously published experiments (Rhoads, Alving, Hiller and Van Slyke, 1934; Van Slyke, Rhoads, Hiller and Alving, 1934a, b). The animals have been in good health for two to three years, and have shown no marked changes in renal function since their preparation.

The dogs were kept on a uniform diet throughout the period of the experiments. The diet consisted of "Purina dog chow," a commercial preparation, supplemented by raw meat, so that the daily intake of protein was 4 to 5 grams per kilo.

All experiments were performed 18 to 20 hours after feeding. When urea and creatinine were given by stomach tube they were dissolved in a quantity of water sufficient to give an intake of 12.5 cc. per kilo body weight, in order to obtain a flow of urine well above the possible augmentation limit of the dog. The dosage was 0.5 gram urea per kilo body weight and 0.25 gram creatinine per kilo. When urea and creatinine were given intravenously the same dosage was used. Inulin and sodium ferrocyanide were administered intravenously, 0.5 gram of each per kilo body weight. The sodium ferrocyanide dosage was calculated on the basis of the anhydrous salt. The crystalline $Na_4Fe(CN)_6 \cdot 12H_2O$ was used. Each substance was dissolved in sterile physiological saline solution. Inulin was made up in 17 per cent solution, sodium ferrocyanide in 15 per cent solution calculated on the dry salt basis. In the majority of the experiments the intravenous injections were given at the rate of 10 cc. per minute, until all the solution was injected (15–20 min.). The clearance periods were then started, with falling plasma concentration curves of the injected substances. In three experiments urea, creatinine, inulin and sodium ferrocyanide were administered intravenously at a constant rate by means of a Woodyatt pump, throughout the time of the experiment which lasted $1\frac{1}{2}$ to 2 hours. The rate of infusion was 1.5 to 2.7 cc. per minute. The total amount of substances given was the same. This procedure gave gradually rising curves for plasma concentrations.

Each experiment included three successive periods of short duration, usually 15 to 40 minutes. The dog was catheterized at the beginning of each experiment and at the end of each period. After the bladder was emptied, it was washed twice with 30 cc. portions of sterile 0.9 per cent sodium chloride solution. The two washings were collected together as

a sample separate from the undiluted urine, as was previously described by Rhoads, Alving, Hiller and Van Slyke (1934). This was done in order to permit separate analyses of urea in urine and washings, from which the volume of urine present in the washings could be calculated and the exact rate of urine output could be determined. Near the middle of each excretion period a blood sample was drawn into an oxalated flask from the renal vein and immediately afterwards another sample was drawn from the femoral artery. Portions were removed for hematocrit determinations, and the rest of the blood was then centrifuged immediately. All analyses were performed on plasma. The plasma concentration of each substance was plotted against time on a large scale, and the concentrations in both arterial and renal venous plasma were interpolated for the middle of each period.

Plasma and urine samples were analyzed for urea, creatinine, inulin, and sodium ferrocyanide. Plasma clearances and renal extraction percentages[5] were calculated for these substances.

Plasma was analyzed rather than whole blood, since it was found that sodium ferrocyanide and inulin were not present in the erythrocytes during these experiments. Creatinine diffuses to and from the cells but does it so slowly that the entire measurable renal fall in blood creatinine concentration occurs in the plasma; in the cells we have observed no fall in creatinine content within the limit of analytical error (Van Slyke, Hiller and Miller, 1935) in the time occupied by the passage of the blood through the kidney and its subsequent centrifugation. Urea, on the other hand, distributes itself instantly between plasma and cells, in equal concentration per unit of water present. This behavior does not affect the clearances, but must be allowed for when urea extraction values are interpreted (Van Slyke, Hiller and Miller, 1935).

Urea was determined in plasma by the gasometric direct urease method (Van Slyke, 1927; Peters and Van Slyke, p. 373, 1932) in the experiments in which ferrocyanide was not administered. One cubic centimeter samples were generally used, but when 1 cc. could not be obtained 0.5 cc. samples were used, and the method was carried out as described previously (Van Slyke, Rhoads, Hiller and Alving, 1934a). Urea was determined in the urine and in the washings separately by the gasometric urease method (Van Slyke, 1927; Peters and Van Slyke, p. 361, 1932). This allowed an exact estimation of the volume output per minute. Sodium ferrocyanide interferes with the action of urease. Consequently when ferrocyanide was administered urea was determined in the plasma and the urine and washings by the manometric hypobromite method (Van

[5] The term "extraction" is borrowed from Dunn, Kay and Sheehan (1931) to indicate the percentage of arterial urea, creatinine, or other solute, that is removed from the blood or plasma as it perfuses the kidney.

Slyke, 1929; Van Slyke and Kugel, 1933; Peters and Van Slyke, p. 379, 1932).

Inulin was determined in plasma and in urine as the increase in reducing substances caused by hydrolysis with acid. From this figure a correction was subtracted for the increase in reducing substances observed when blood drawn before inulin injection was treated in the same manner. In order to lower the non-glucose reducing substances in the blood as far as possible cadmium hydroxide, introduced by Fujita and Iwatake (1931) was used as a protein precipitant. This precipitant was found to remove from the normal dog plasma almost completely the material which yields reducing substances on acid hydrolysis. The cadmium further removes ferrocyanide from plasma in those experiments in which it is present. The cadmium treatment did not remove any inulin. The details of the procedure were as follows:

The precipitation of proteins in plasma was carried out as described by Fujita and Iwatake (1931) for blood, with a slight modification for plasma. Into a 20 cc. volumetric flask were measured 1 cc. of plasma, 10 cc. of water, 4 cc. of the acid cadmium sulfate solution,[6] and 0.5 cc. of 1.1 N sodium hydroxide. The contents were diluted to 20 cc. with water, centrifuged, and filtered through a small filter paper which had been previously washed free of reducing substances and dried. Two cubic centimeters of this filtrate are equivalent to 0.1 cc. of plasma. We analyzed 1 or 2 cc. portions of the filtrate by the ferricyanide reduction method of Hagedorn and Jensen (1923) both before and after hydrolysis.

A. The *free reducing substances* (chiefly glucose) were determined by measuring 1 or 2 cc. of the filtrate into a 50 cc. centrifuge tube, adding 14 or 13 cc., respectively, of water, and 2 cc. of the Hagedorn and Jensen ferricyanide reagent, and following the method of Hagedorn and Jensen exactly from this point.

B. To determine the *total reducing substances* after acid hydrolysis 1 or 2 cc. of the filtrate were pipetted into a 50 cc. centrifuge tube, and 1 cc. of normal hydrochloric acid and sufficient water to make 10 cc. were added. Each tube was covered with a glass bulb to prevent undue evaporation and placed in a water bath at 80°C. for two hours. The tube was then cooled to room temperature, and the acid was neutralized with 1 cc. of normal sodium hydroxide. Four cubic centimeters of water and 2 cc. of the Hagedorn-Jensen ferricyanide reagent were added, and the sugar analysis was carried out from this point according to Hagedorn and Jensen.

C. The *naturally occurring hydrolyzable reducing substances* were determined by drawing a sample of blood from the animal just before the injection of inulin at the beginning of the experiment and analyzing it

[6] The acid cadmium sulfate solution contained 13 grams of crystalline cadmium sulfate, 3 $CdSO_4 \cdot 8\ H_2O$ and 63.5 cc. of normal sulfuric acid in a volume of 1 liter.

for reducing substances before and after hydrolysis. The *increase* caused by hydrolysis, indicated as C in the calculation formula below, was always found to be under 5 mgm. per 100 cc. of plasma.

Inulin was calculated

$$\text{Inulin} = B - A - C$$

where A, B and C represent the reducing values, calculated as glucose, found by procedures A, B and C.

For determination of inulin in urine aliquot portions of urine and washings were united. These mixed urine specimens were similarly treated with the cadmium precipitating reagents, and the filtrate was analyzed by the Hagedorn and Jensen method before and after hydrolysis. A control urine was obtained before the beginning of the experiment and analyzed in the same manner as the control plasma for the C correction.

Ferrocyanide was determined colorimetrically. The proteins were precipitated from the plasma with tungstic acid by the method of Folin and Wu (1919), using half the quantities of sodium tungstate and sulfuric acid that are used for whole blood. An amount of filtrate containing between 0.8 and 1.3 mgm. of sodium ferrocyanide was measured into a 25 cc. volumetric flask, and the Prussian blue color developed with 5 cc. of the acid ferric sulfate reagent described by Folin (1929) for his blood sugar method (Folin, 1928). The procedure of Folin for making the ferric sulfate solution was followed exactly except that the oxidation with potassium permanganate was omitted. After standing 5 minutes the solution was diluted to volume with water, and the color read against a standard made up in a similar manner, but which contained 1 mgm. of sodium ferrocyanide and 5 cc. of tungstate filtrate from a control plasma taken from the animal just before injection of the sodium ferrocyanide. If the volumes of the unknown solutions at this point in the determination were greater than in the standard, water was added to the standard to make all volumes approximately equal before adding 5 cc. of the acid ferric sulfate reagent to develop the Prussian blue color. The standard solution, containing 0.5 mgm. of the anhydrous salt per cubic centimeter was made from J. T. Baker's sodium ferrocyanide C.P. The standard was checked by titration with potassium permanganate or ceric sulfate.

Sodium ferrocyanide was recovered quantitatively by the method described when added to plasma in amounts between 70 and 200 mgm. per 100 cc.

The mixed specimens of urine and washings were analyzed similarly for ferrocyanide by diluting so that 5 cc. would contain approximately 1 mgm. of sodium ferrocyanide, and using an amount of the diluted urine which could be read against the 1 mgm. standard in the 25 cc. volume.

Creatinine was determined in the plasma by applying the method of

Folin and Wu (1919), to the same cadmium filtrate that was prepared for the determination of inulin. To duplicate 3 cc. portions of each filtrate 2 cc. of alkaline picrate solution were added. After the mixtures had stood not less than 10 nor more than 20 minutes they were compared in a compensating Hastings colorimeter with standard creatinine solution. The cups E and E′ (fig. 88, p. 802, Peters and Van Slyke, 1932) were filled with the standard and the unknown respectively. Cups F and F′ were both filled with a solution made by diluting two parts of the alkaline picrate solution with three parts of water. Cups G and G′ were left empty. This arrangement causes the light to pass through equal layers of alkaline picrate on each side, so that the differences in observed color are due entirely to the colored product formed from the creatinine.

Duplicate solutions of each plasma filtrate were routinely prepared, because they frequently developed colors differing by several per cent. For this behavior we could not determine the cause. When the duplicates showed a difference of more than 4 per cent a third sample of filtrate was analyzed, if sufficient filtrate was available.

Creatinine was recovered by this procedure when added to plasma in amounts varying between 5 and 30 mgm. per 100 cc.

Urine was diluted to bring the creatinine content down to 0.1 to 0.3 mgm. per cubic centimeter, and was similarly analyzed in duplicate.

The alkaline picrate solution used was made by mixing 5 volumes of saturated picric acid solution with one volume of 10 per cent NaOH solution. The picric acid was purified by the method of Benedict (1929).

Calculation of plasma clearances. The urea clearance was calculated as described in a previous paper (Rhoads, Alving, Hiller and Van Slyke, 1934), except that in the present paper B represents concentration in plasma instead of in whole blood. Sodium ferrocyanide, inulin, and creatinine clearances were also calculated according to the same formula. All clearances were calculated per square meter of body surface as described in the same paper.

Calculation of the percentage of substances extracted from the plasma by the kidney. This value has been termed "extraction percentage," (Van Slyke, Rhoads, Hiller and Alving, 1934a), and is calculated as

$$E = \frac{A - R}{A} \times 100$$

E = extraction percentage; A = concentration of substance in arterial plasma; R = concentration of substance in renal venous plasma.

RESULTS. *Clearances in rabbits.* Five comparative clearances were obtained in two experiments with rabbits. In the first experiment the exact technique of ferrocyanide administration employed by Gersh and Stieglitz in their study on rabbits was employed so that one may correlate

the ferrocyanide clearances directly with their histological results. In the second experiment somewhat larger amounts of ferrocyanide were injected to facilitate the analyses. The simultaneous clearances of ferrocyanide, inulin, creatinine and urea are given in table 1. There is close agreement between ferrocyanide and creatinine clearances in each experiment. The inulin clearance is also of the same order of magnitude except in the first period in experiment 2. In each case urea showed a lower clearance than ferrocyanide or creatinine.

Clearances in dogs. A typical experiment is presented in tables 2 and 3 and in figures 1 and 2. In figure 1 the clearances have been plotted as a function of time. In this particular experiment all the substances were injected intravenously at a constant rate throughout the entire time, thereby giving a continuous increase in their concentrations. As in the case of the rabbit there is found a close correspondence in the simultaneously determined clearances of ferrocyanide, creatinine and inulin. The urea clearance, as usual, is lower. The rise in all clearance values during

TABLE 1

Comparative clearances of ferrocyanide, creatinine, inulin and urea in the rabbit

TYPE OF CLEARANCE	RABBIT I		RABBIT II		
	Period 1	Period 2	Period 1	Period 2	Period 3
Ferrocyanide	49	19	37	31	10
Creatinine	54	21	38	31	9
Inulin	54	18	22	26	9
Urea	19	6	23	15	2

the course of this experiment was probably caused by increased blood flow through the kidney and not by the changing plasma concentration. Van Slyke, Rhoads, Hiller, and Alving (1934b) have demonstrated with urea that the clearance varies as a linear function of the renal blood flow, but is completely independent of the blood urea concentration.

The results of the entire series of clearances in dogs have been summarized in table 4 in the form of ratios.[7] The ratio given is the mean of the ratios of the individual, simultaneous clearances. The standard deviation is calculated as

$$\sqrt{\frac{\Sigma\delta^2}{N}}$$

[7] The absolute values of the plasma clearances when calculated on a cubic centimeter per square meter basis lie chiefly between 45 and 95 for ferrocyanide, creatinine, and inulin, and 25 to 55 for urea. These figures should not be compared with those for other animals unless it be noted that they represent clearances on dogs possessing only single kidneys.

where δ is the deviation from the mean, and N is the number of observations. The mean observed clearance ratios of ferrocyanide to creatinine, ferrocyanide to inulin, and inulin to creatinine are all as near to unity as could be expected if the true ratio is unity, and the degree of variability in observation indicated by the standard deviation is taken into account. Our results for the clearance ratio of inulin to creatinine also agree as

TABLE 2

Plasma concentrations in an experiment in which sodium ferrocyanide, inulin, creatinine and urea were injected intravenously by constant infusion with a Woodyatt pump during the entire course of the experiment. Dog D7 (one kidney explanted, one removed)

The values given are those interpolated for the mid-point of each period of urine collection.

PERIOD NUMBER	PLASMA CONCENTRATION							
	Sodium ferrocyanide		Inulin		Creatinine		Urea N	
	Arterial	Renal venous	Arterial	Renal venous	Arterial	Renal venous	Arterial	Renal venous
	mgm. per cc.	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc*
1	0.633	0.480	0.552	0.365	0.175	0.140	0.447	0.430
2	1.220	0.987	0.938	0.745	0.347	0.274	0.575	0.520
3	1.575	1.295	1.075	0.908	0.406	0.313	0.663	0.603

TABLE 3

Urine analyses of experiment on dog D7. The true urine volume is the volume of urine collected plus the calculated volume of urine in the washings

PERIOD NUMBER	LENGTH OF PERIOD	VOLUME				URINE CONCENTRATION			
		Urine	Washings	Mixed urine (urine + washings)	True urine volume	Sodium ferrocyanide per cc. mixed urine	Inulin per cc. mixed urine	Creatinine per cc. mixed urine	Urea N per cc. true urine
	minutes	*cc.*	*cc.*	*cc.*	*cc.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	26.0	25.0	67.0	92.0	28.3	6.26	5.52	1.98	9.06
2	28.5	61.0	65.0	126.0	69.9	13.43	10.41	3.53	6.73
3	19.5	57.8	73.5	131.3	71.3	12.75	8.57	3.65	5.96

closely as could be expected with those of Richards, Westfall and Bott (1934) who, in 34 comparative clearances on the dog obtained a mean ratio of 1.07, with a standard deviation of 0.20,[8] and with those of Shannon (1935b).

[8] This standard deviation was calculated from the data of Richards, Westfall and Bott by the same formula used for our results.

The degree of variability of the ratios of creatinine, inulin, ferrocyanide from unity may in large degree be attributed to experimental error of the procedures involved. Neither the colorimetric analysis of creatinine nor that of ferrocyanide is a precise method, and in calculation of the clearances the errors in analyses of urine and plasma are additive. The plasma inulin, being determined by difference of sugar contents found before and after hydrolysis, is also not a very accurate determination, especially when the inulin is low compared with the glucose present. The plasma results in particular suffer in accuracy when the concentrations determined are low, as they were in the initial periods of the experiments with steady injection by the Woodyatt pump, and in the last periods of experiments in which all the materials were injected at the start. The use of interpolation curves for estimating plasma concentration at the mid-point of time for each period adds somewhat to the error, as does also the assumption that the plasma concentration at the mid-point represents the mean for the period. Our most constant clearance ratios were obtained in the experiments with the Woodyatt pump. The results of one of these experiments have been given in figure 1.

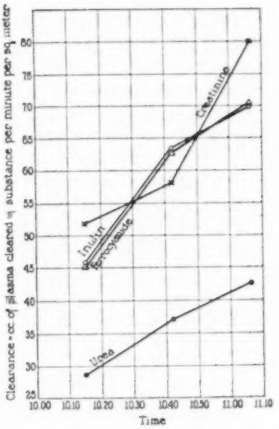


Fig. 1. The comparative clearances of ferrocyanide, creatinine, inulin, and urea in one experiment on dog D-7.

Relation of clearance to plasma concentration. It is axiomatic that the clearance ratios of substances which represent the true glomerular filtrate must be independent of their plasma concentrations. In observing simultaneous creatinine and inulin clearances, Shannon has found in both the dogfish (1934) and in man (1935a), that the ratio of the two clearances does not remain constant at unity with changes in plasma concentration. On the contrary the creatinine clearance became considerably higher than the inulin clearance when plasma creatinine concentration fell to low levels. This was taken as evidence of the probable partial tubular secretion of creatinine, the part secreted by the tubules becoming greater, in comparison with the part filtered in the glomeruli, when plasma concentration was lower. However, as has been suggested by Shannon, the interpretation depends on the unproved assumption that the inulin clearance represents the true glomerular filtrate in the dogfish and in man.

It is of interest to see whether this effect is ever found in our experiments on dogs, where we are reasonably sure that ferrocyanide clearance

represents true filtrate. From inspection of figure 3 one may readily see that this effect is *not* observed in the dog, and that *change of creatinine plasma concentration from 10 to 60 mgm. per 100 cc. does not change the clearance ratio of ferrocyanide and creatinine.* This is reasonably good evidence that creatinine is excreted in the same way as ferrocyanide in the dog. That way is, we believe, by glomerular filtration.

TABLE 4

Ratios of clearances of ferrocyanide, creatinine, inulin and urea in dogs

CLEARANCE RATIO	NUMBER OF DETERMINATIONS	MEAN RATIO	STANDARD DEVIATION OF RATIO
Ferrocyanide/creatinine	27	0.96	0.18
Ferrocyanide/inulin	14	1.04	0.13
Inulin/creatinine	24	0.97	0.14
Ferrocyanide/urea	32	1.74	0.37
Inulin/urea	24	1.91	0.45

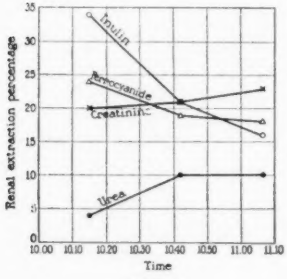


Fig. 2

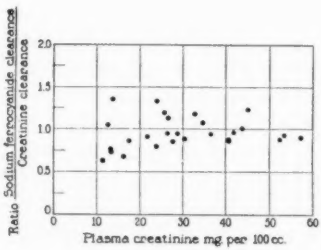


Fig. 3

Fig. 2. The comparative renal extraction percentages during constant intravenous injection of ferrocyanide, creatinine, inulin, and urea in the experiment recorded in figure 1.

The agreement of results was not so good in the first period as in the later periods probably because the concentration of substances in the plasma was low and therefore the errors in analyses were greater (see table 2).

Fig. 3. Relationship between the ratio of ferrocyanide clearance to creatinine clearance and the concentration of creatinine in the plasma.

This conclusion is in agreement with that reached by Shannon in a study of the dog (1935b), in which the clearance ratios of creatinine to inulin were found to be independent of plasma concentration, in contrast to his findings in man and the dogfish.

Extraction percentages of ferrocyanide, inulin, creatinine and urea. The extraction percentages obtained at the same time as the clearances are summarized in table 5 and figure 4, again demonstrating the similarity in behavior of ferrocyanide, inulin, and creatinine.

Figure 4 presents evidence that *there is no observable dependence of the extraction percentages on plasma concentrations of any of these substances* in our experiments in dogs. This figure gives the results of a number of experiments on different animals. The variability of the extraction

TABLE 5

Extraction of ferrocyanide, creatinine, inulin and urea from blood plasma by the dog kidney

SUBSTANCE	NUMBER OF DETERMINATIONS	MEAN EXTRACTION PERCENTAGE	STANDARD DEVIATION
Ferrocyanide	25	18.8	5.5
Creatinine	36	19.9	3.8
Inulin	21	22.3	7.9
Urea	37	8.3†	2.0

† The observed decrease of 8.3 per cent in urea concentration of renal vein plasma as compared with arterial plasma indicates in fact an excretion of about 12.8 per cent of the arterial plasma urea (see Van Slyke, Hiller and Miller, 1935). For the other three substances, which do not diffuse significantly between cells and plasma during the time intervals involved, the decrease in plasma concentration indicates the actual percentage of arterial plasma content which is excreted.

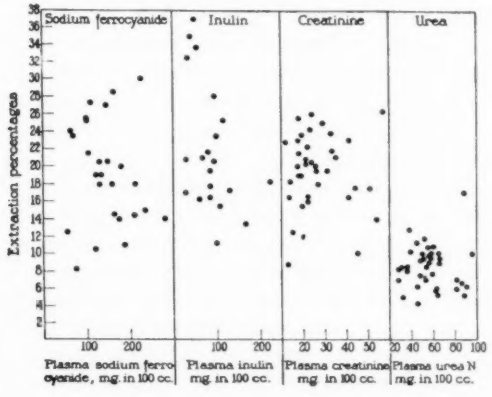


Fig. 4. Relationship between the extraction percentages of ferrocyanide, creatinine, inulin, and urea and their respective concentrations in the plasma.

percentages is doubtless in part physiological, but, in the cases of creatinine, inulin and ferrocyanide, it must be in part attributed to the error involved in determining the extraction as the difference between analyses of arterial and renal venous plasma, neither of which is accurate within several per cent. For example, if the true plasma creatinine extraction

is 20 per cent, an error of −5 per cent in the analysis of the arterial plasma combined with an error of +5 per cent in the analysis of renal venous plasma would lower the observed percentage extraction to 10; whereas similar errors in the opposite direction would raise it to 30. Errors of this magnitude in the methods used for creatinine, ferrocyanide and inulin are possible. The exceptionally high inulin extractions in 4 observations (fig. 4), including the first in figure 2, occurred in periods when the plasma inulin content was low, and may be entirely due to the error involved in determining small concentrations of inulin by the method of difference.

Figure 2 demonstrates the identity, within the limits of experimental error, of the extraction percentages of inulin, creatinine, and ferrocyanide during a single experiment in which these substances and urea were injected at a constant rate throughout the experiment. There was a steady increase in the plasma concentrations of all 4 compounds (table 2), plasma inulin, creatinine, and ferrocyanide being each about 2.5 times greater in the last period than in the first. Yet their extraction percentages showed no significant variation. (The high initial extraction for inulin may be attributed to the especially high error of determining small concentrations of this substance in the plasma by difference.)

Discussion. *Does the ferrocyanide clearance represent the true glomerular filtrate?* The independent evidence, that ferrocyanide is handled by the kidney in such a manner that its clearance may serve as a measure of true glomerular filtrate, consists of the histochemical observations made by Gersh and Stieglitz in the rabbit, and by Gersh (personal communication) in the dog and other mammals. That these studies indicate the true course of the ferrocyanide excretion seems to us fairly certain because of the following considerations:

1, Gersh's technique prevents diffusion of soluble salts before staining; 2, the Prussian blue staining method is sufficiently sensitive; 3, the technique employed will detect ferrocyanide in the tubular epithelium when it is present, as demonstrated by Gersh and Stieglitz in the case of the "storage phenomenon," and 4, no excretion of ferrocyanide was noted when the filtration pressure was markedly reduced by section of the spinal cord. Additional proof of somewhat less direct nature comes from the fact that the same method used by Gersh in the study of phenol red excretion (1934) showed excretion by the tubular cells without marked glomerular filtration. This result is in accord with the established physiological measurements of Marshall (1931) and indicates that the histological findings of Gersh are not due to artefacts.

That ferrocyanide is excreted without damage to the renal elements is indicated by the following evidence. During the experiments, the urea clearance was as high as in previous control observations or higher, and no hematuria, either gross or microscopic, could be observed. Further,

when the urea clearance was followed for several weeks after the injection of sodium ferrocyanide, it was found to be entirely normal; and no chronic hematuria, albuminuria or cylindruria has been noted.

We shall assume in the following discussion and calculations that the ferrocyanide clearance in the dog is a measure of the true glomerular filtrate.

The mechanism of creatinine and inulin excretion. Evidence that creatinine and inulin are excreted by a process of pure filtration comes from data which have been presented on both clearance and extraction values. The clearances of these two compounds have been shown to be identical with that of ferrocyanide. The range of plasma concentrations studied has been great enough, it is believed, to eliminate any chance coincidence. In addition it has been found that all three compounds have essentially the same extraction percentage, viz., about 20, and that this value remains independent of plasma concentration.

It is of interest to compare these extraction percentages with those found by Marshall (1931) in his study of phenol red excretion in the dog. In the case of phenol red the extraction percentage varied from 34 to 75 per cent, amounts apparently too great to be due solely to filtration. Also Marshall observed that the extraction of phenol red varied inversely with the plasma concentration.

If, as seems probable, ferrocyanide is excreted entirely by filtration, the fact that the clearances and extraction percentages of creatinine and inulin agree so well with those of ferrocyanide, is evidence in favor of the probability that, in the dog, creatinine and inulin are also excreted solely by filtration.

The mechanism of urea excretion. The clearance and the extraction percentage of urea are definitely lower than of ferrocyanide. This difference may be most easily explained by assuming that urea is filtered in the same proportion as ferrocyanide, but that part of the filtered urea is reabsorbed in the tubules. It is difficult to believe that urea does not pass the glomerular capillaries as easily as ferrocyanide because: 1, urea is known to diffuse throughout the body, intracellularly as well as extracellularly, with peculiar ease; 2, if urea did not pass the glomerulus freely it would set up an osmotic force in the plasma greater than the effective filtration pressure (Cushny, 1926b). If it be granted that urea filters into Bowman's capsule as easily as ferrocyanide, then reabsorption of urea must be assumed from the fact that the clearance of urea is lower than that of ferrocyanide. From the average ratio, 1.74, of the ferrocyanide to urea clearance (table 4) one may calculate that the average reabsorption of urea under the conditions of our experiments amounted to

$$\frac{1.74 - 1.00}{1.74} \times 100 = 43 \text{ per cent of the filtered urea.}$$

The possibility does exist that the reabsorption may have been greater than this, and that some urea was secreted by the tubular epithelium. This seems unlikely because it would necessitate passage of urea through the tubular epithelium in both directions at the same time. This state of affairs could exist only if there were a high degree of specialization in different portions of the tubules, so that absorption of urea could take place at one point and secretion at another. For the time being it seems logical to assume that urea is excreted in the dog's kidney by a process of glomerular filtration and tubular reabsorption.

Proportion of plasma water filtered. It appears probable that the approximate 20 per cent extraction of ferrocyanide, inulin and creatinine from the plasma indicates filtration of 20 per cent of the arterial plasma water into the glomerular capsules. This probability follows from the following reasoning:

According to present conceptions of excretion, water and the filterable solutes which it contains are filtered in the same proportions in which they exist in the plasma. If 20 per cent of the plasma water is filtered, an equal percentage of the ferrocyanide, etc., in each cubic centimeter of arterial plasma is filtered with the water. Richards and his collaborators (Richards, 1929; Wearn and Richards, 1925; Richards, Livingston and Freeman, 1930; Walker and Elsom, 1931; Walker and Reisinger, 1933; Bordley, Hendrix and Richards, 1933; Bordley and Richards, 1933, and Westfall, Findley and Richards, 1934) have shown that filterable solutes and water exist in the same proportions in the glomerular filtrate of the frog as in the plasma, and it appears reasonable to assume that the same type of filtration occurs in the glomerulus of the dog. In accordance with this conception the immediate effect of filtration on the plasma therefore, is to reduce its volume, without affecting the concentrations of its filterable solutes per unit of plasma water present.

In the tubules, however, nearly all of the water is reabsorbed, so that the plasma volume is restored, and the *concentrations* of the non-reabsorbed, filterable solutes in the renal vein plasma become lowered below arterial concentrations by a percentage nearly equal to the percentage of water filtered. The "percentage extractions" which we have calculated (table 5) represent such percentage decreases calculated from observed concentrations in the plasma of arterial and renal vein blood.

The necessarily slight effect of excretion on the plasma volume of blood in the renal vein is indicated by the following example. Under ordinary conditions 300 cc. of blood, with 175 cc. of plasma, may flow through the kidneys of a dog per minute (Van Slyke, Rhoads, Hiller and Alving, 1934a), while only 0.5 cc. of urine is excreted. The volume of excreted urine would diminish the plasma volume by only 1 part in 300.

Relation between renal blood flow and the plasma clearance of ferrocyanide, inulin or creatinine. The ferrocyanide, inulin and creatinine removed from the blood by the kidneys are taken entirely from the plasma and not from the cells, as already indicated. The plasma clearance value, C, of any excreted substance represents by definition the cubic centimeters of plasma containing the amount of that substance which is excreted in one minute. If $1/n$ represents the fraction of the plasma content of the substance which is removed during perfusion of the kidney, then $n\ C$ is the volume of plasma per minute which perfuses the kidneys.

In the cases of creatinine, inulin, and ferrocyanide, the average percentage extraction is 20, so that the fraction $1/n = \frac{1}{5}$, and the renal plasma flow per minute is $5 \times C$. Hence in a normal dog the average volume of plasma perfusing the kidneys per minute is approximately 5 times the plasma clearance of creatinine, inulin, or ferrocyanide. The corresponding renal flow of whole blood would be calculated as $n\ C \times \frac{1}{V_p}$, where V_p represents the volume of plasma in one volume of whole blood.

SUMMARY. In the dog sodium ferrocyanide, inulin, and creatinine have been found to show the same excretory behavior in the following respects:

a. Their plasma clearances are approximately equal.

b. Their percentage extractions from the plasma are also approximately equal, averaging approximately 20.

c. Their clearances and extraction percentages are independent of plasma concentration.

The clearance of urea has been found to average 0.57 of the clearances of the above 3 substances, and to be similarly independent of plasma concentration.

CONCLUSIONS

The above observations, taken with the histochemical evidence of Gersh that ferrocyanide is excreted entirely by glomerular filtration, appear to support the filtration-reabsorption theory of renal excretion.

Interpreted in accord with this theory, the observations lead to the following conclusions:

a. The plasma clearance of ferrocyanide, inulin, or creatinine in the dog is equal to the glomerular filtrate.

b. From each cubic centimeter of plasma entering the glomeruli on the average $\frac{1}{5}$ of the water and of the filterable solutes is filtered.

c. The volume of blood *plasma* flowing per minute through the kidneys of dogs averages about 5 times the glomerular filtrate, or the plasma clearance, of sodium ferrocyanide, inulin, or creatinine.

d. Of the filtered urea, on the average about 43 per cent is reabsorbed.

REFERENCES

BENEDICT, S. R. J. Biol. Chem. **82:** 1, 1929.

BORDLEY, J., 3RD, J. P. HENDRIX AND A. N. RICHARDS. J. Biol. Chem. **101:** 255, 1933.

BORDLEY, J., 3RD AND A. N. RICHARDS. J. Biol. Chem. **101:** 193, 1933.

CHASIS, H., N. JOLLIFFE AND H. W. SMITH. J. Clin. Invest. **12:** 1083, 1933.

CUSHNY, A. R. The secretion of the urine. 2nd ed., Longmans, Green and Co., Ltd., London, 1926a.

Ibid. p. 61, 1926b.

DUNN, J. S., W. W. KAY AND H. L. SHEEHAN. J. Physiol. **73:** 371, 1931.

FOLIN, O. J. Biol. Chem. **77:** 421, 1928.

J. Biol. Chem. **81:** 231, 1929.

FOLIN, O. AND H. WU. J. Biol. Chem. **38:** 81, 1919.

FUJITA, A. AND D. IWATAKE. Biochem. Ztschr. **242:** 43, 1931.

GERSH, I. Anat. Rec. **53:** 309, 1932.

This Journal **108:** 355, 1934.

GERSH, I. AND E. J. STIEGLITZ. Anat. Rec. **58:** 349, 1934.

HAGEDORN, H. C. AND B. N. JENSEN. Biochem. Ztschr. **135:** 46, 1923.

JOLLIFFE, N. AND H. CHASIS. This Journal **104:** 677, 1933.

JOLLIFFE, N., J. A. SHANNON AND H. W. SMITH. This Journal **100:** 301, 1932.

MARSHALL, E. K., JR. This Journal **99:** 77, 1931.

MARSHALL, E. K., JR. AND A. GRAFFLIN. Johns Hopkins Hosp. Bull. **63:** 205, 1928.

MØLLER, E., J. F. MCINTOSH AND D. D. VAN SLYKE. J. Clin. Invest. **6:** 427, 1928.

PETERS, J. P. AND D. D. VAN SLYKE. Quantitative clinical chemistry, Vol. II, 1932. The Williams & Wilkins Co., Baltimore.

REHBERG, P. B. Biochem. J. **20:** 447, 1926.

RHOADS, C. P. Science **73:** 417, 1931.

This Journal **109:** 324, 1934.

RHOADS, C. P., A. S. ALVING, A. HILLER AND D. D. VAN SLYKE. This Journal **109:** 329, 1934.

RICHARDS, A. N. Methods and results of direct investigations of the function of the kidney. The Williams & Wilkins Co., Baltimore, 1929.

RICHARDS, A. N., A. E. LIVINGSTON AND B. FREEMAN. J. Biol. Chem. **87:** 467, 1930.

RICHARDS, A. N., B. B. WESTFALL AND P. A. BOTT. Proc. Soc. Exper. Biol. and Med. **32:** 73, 1934.

SHANNON, J. A. J. Cell. and Comp. Physiol. **5:** 301, 1934.

J. Clin. Invest. **14:** 403, 1935a.

This Journal **112:** 405, 1935b.

SHANNON, J. A., N. JOLLIFFE AND H. W. SMITH. This Journal **102:** 534, 1932.

SHANNON, J. A. AND H. W. SMITH. J. Clin. Invest. **14:** 393, 1935.

VAN SLYKE, D. D. J. Biol. Chem. **73:** 695, 1927.

J. Biol. Chem. **83:** 449, 1929.

VAN SLYKE, D. D., A. HILLER AND B. F. MILLER. This Journal **113:** 629, 1935.

VAN SLYKE, D. D. AND V. H. KUGEL. J. Biol. Chem. **102:** 489, 1933.

VAN SLYKE, D. D., C. P. RHOADS, A. HILLER AND A. S. ALVING. This Journal **109:** 336, 1934a.

This Journal **110:** 387, 1934b.

WALKER, A. M. AND K. A. ELSOM. J. Biol. Chem. **91:** 593, 1931.

WALKER, A. M. AND J. A. REISINGER. J. Biol. Chem. **101:** 223, 1933.

WEARN, J. T. AND A. N. RICHARDS. J. Biol. Chem. **66:** 247, 1925.

WESTFALL, B. B., T. FINDLEY AND A. N. RICHARDS. J. Biol. Chem. **107:** 661, 1934.

THE DISTRIBUTION OF FERROCYANIDE, INULIN, CREATININE AND UREA IN THE BLOOD AND ITS EFFECT ON THE SIGNIFICANCE OF THEIR EXTRACTION PERCENTAGES

DONALD D. VAN SLYKE, ALMA HILLER AND BENJAMIN F. MILLER[1]

From the Hospital of the Rockefeller Institute for Medical Research, New York, N. Y.

Received for publication July 17, 1935

In the preceding paper observations on the plasma clearances and extraction percentages of ferrocyanide, inulin, creatinine, and urea have been reported. In order to define the significance of the extraction percentages and their relations to the clearances, it proved necessary to ascertain the distributions and diffusion rates between cells and plasma of the substances studied. This paper presents a study of the distribution and diffusion rate factors, and their effects on the interpretation of observed percentage extractions from plasma and whole blood. Investigations of the distribution of urea between red cells and plasma (Gad-Andresen, 1921; Wu, 1932; Folin and Berglund, 1922, and Berglund, 1922) have demonstrated that this substance, because of its complete diffusibility, is distributed in the same proportion as the water content, about 75 to 80 per cent as much in cells as in plasma per unit volume. Reports by earlier investigators on the distribution of creatinine between cells and plasma (Hunter and Campbell, 1917; Wilson and Plass, 1917, and Wu, 1922) indicate that this substance is about equally distributed between cells and plasma. No published data have been found on the distribution of sodium ferrocyanide or inulin, or on the relative ratio of diffusion of the 4 substances under consideration.

EXPERIMENTAL. The distribution of urea and creatinine between cells and plasma in the blood of the dog was determined by means of experiments both *in vitro* and *in vivo*. The distribution of inulin and sodium ferrocyanide was determined *in vivo*.

The *experiments in vitro* were performed by equilibrating dog blood at 38°C. after addition of urea and creatinine. One portion of blood was centrifuged before addition of urea and creatinine, and cells and plasma were analyzed for these substances. These control analyses determined the distribution of the normal amounts found in dog blood. To a 50 cc. portion of the same sample of blood were added 5 cc. of a solution containing 0.2 per cent creatinine and 1 per cent urea, so that the concentra-

[1] National Research Council Fellow in Medicine.

tion of added creatinine in the mixture was 18.18 mgm. per 100 cc., and the concentration of added urea was 91 mgm. One portion of this mixture was centrifuged immediately, the remainder was placed in an incubator at 38°C. and shaken gently every 5 minutes. Portions were removed after 30 and 60 minutes and centrifuged. Both cells and plasma were analyzed for creatinine and urea in all portions.

The *experiments in vivo* were performed after intravenous injection of the substances under observation into dogs in which one kidney had been explanted by the technique of Rhoads (1931, 1934) and the other kidney had been removed. Samples of arterial and renal venous blood were removed at varying intervals after injection. The plasma and cells were immediately separated by centrifugation and analyzed for the different substances injected. In one experiment urea and creatinine were administered by stomach tube.

METHODS. The *separation of cells and plasma* in the experiment performed *in vitro* and in the experiments on inulin and ferrocyanide *in vivo* was effected by centrifugation at 3000 revolutions per minute for 40 minutes. After the plasma layer was removed the layer of white cells together with a layer about 1 mm. thick of red cells was removed by suction applied to a tube drawn out to a fine point. The remaining cells were well mixed with a fine stirring rod before samples were removed for analysis. In the experiments on the distribution of creatinine and urea *in vivo* (figs. 2 and 3) the separation of cells and plasma was effected by centrifugation at 20,000 revolutions per minute for 10 minutes. This procedure reduced the total time between the withdrawal of blood from the animal and the separation of plasma from cells to 20 minutes.

The *plasma* was analyzed for ferrocyanide, inulin, creatinine and urea by the methods described in the preceding paper by Van Slyke, Hiller and Miller (1935). Urea was determined by the direct urease method, since ferrocyanide was not injected in the experiments in which urea was determined.

The *cells* were analyzed for urea in the same manner as the plasma. In order to obtain uniform samples a portion of the cells was measured with a pipette calibrated "to contain" into an equal volume of water which laked the cells.

The *ferrocyanide* determinations in the cells were made after precipitation of the protein in the following way: 15 cc. of water were measured into a centrifuge tube. The cells were delivered from a pipette, calibrated to contain 1 cc., into the 15 cc. of water, and the pipette washed by drawing the water up into it repeatedly. After complete hemolysis 2 cc. of 10 per cent sodium tungstate and 2 cc. of $\frac{2}{3}$ normal sulfuric acid were added. The protein precipitate was centrifuged, and the supernatant fluid was filtered through a small filter paper. Since the cell filtrates

developed no perceptible Prussian blue color with ferric sulfate, and since we had no method for determining such small amounts of ferrocyanide, the cell filtrates were analyzed by adding to both standard and unknown 1 mgm. of sodium ferrocyanide in the final volume of 25 cc. To each standard was added 10 cc. of a control filtrate made from cells taken before injection of ferrocyanide into the animal. The unknowns were made by measuring into 25 cc. volumetric flasks 10 cc. portions of the cell filtrates. The addition of 5 cc. of ferric sulfate and dilution to 25 cc. was the same as in the plasma analysis.

For *inulin and creatinine* analysis the cells were prepared by precipitation of proteins with cadmium hydroxide. To 13 cc. of water in a centrifuge tube was added 1 cc. of cells in the same manner described for ferrocyanide determination. After complete hemolysis 4 cc. of a cadmium sulfate solution, four times as concentrated as that used for plasma, were mixed with the laked cells.[2] After 5 minutes 2 cc. of 1.1 normal sodium hydroxide were added, the resulting suspension was well mixed, and was centrifuged. The supernatant fluid was filtered through a small filter paper, which had been previously washed free of reducing substances and dried. Inulin and creatinine were determined in the filtrates as described for plasma in the preceding paper (Van Slyke, Hiller, and Miller, 1935).

RESULTS. *1. Distribution of sodium ferrocyanide between plasma and cells in vivo.* That sodium ferrocyanide does not penetrate into the red cells is shown clearly by an experiment (table 1) where plasma and cells were analyzed after injection of the ferrocyanide.

2. Distribution of inulin between plasma and cells in vivo. The behavior of inulin is the same as sodium ferrocyanide, as demonstrated by the results of a similar experiment, which are shown in table 2. The amounts of inulin found in the cells are within the limits of the error in the method of determining inulin by difference.

3. Distribution of creatinine and urea between plasma and cells in vitro. The results of an experiment in which urea and creatinine were added to dog blood and equilibrated at 38°C. are given in table 3.

Creatinine, in contrast to urea, diffuses very slowly from plasma to cells *in vitro*. The diffusion is so slow that we may assume that plasma and cells separated by centrifugation immediately after blood is drawn have, within the limits of analytical error, the same creatinine content as at the moment when drawn from the blood vessels.

4. Distribution of creatinine and urea between plasma and cells in vivo. The experiment shown in figure 1 illustrates the manner in which the creatinine equilibrium is established *in vivo*. Two experiments have been performed in which the distribution between plasma and cells of both

[2] The acid cadmium sulfate solution contained 5.2 grams of crystalline cadmium sulfate, 3 $CdSO_4 \cdot 8\ H_2O$, and 25.4 cc. of normal sulfuric acid in a volume of 100 cc.

creatinine and urea in *arterial* and *renal venous* blood has been determined. These results are shown in figures 2 and 3.

Figures 2 and 3 show most strikingly that the extraction of creatinine by the kidneys is entirely from the plasma since, while the plasma loses

TABLE 1

The distribution of sodium ferrocyanide between plasma and cells in vivo

TIME AFTER INJECTION OF FERROCYANIDE	CONCENTRATION OF SODIUM FERROCYANIDE PER CC.	
	Plasma	Cells
minutes	*mgm.*	*mgm.*
0	0	0
16	2.155	0
36	1.654	0

TABLE 2

The distribution of inulin between plasma and cells in vivo

TIME AFTER INJECTION OF INULIN	CONCENTRATION OF INULIN PER CC.	
	Plasma	Cells
minutes	*mgm.*	*mgm.*
0	0	0
15	0.932	0.089
32	0.579	0.000
54	0.391	0.052

TABLE 3

The distribution of creatinine and urea between plasma and cells in vitro

TIME OF SAMPLING AFTER ADDITION OF UREA AND CREATININE	CREATININE CONCENTRATION PER CC.			UREA N CONCENTRATION PER CC.		
	Plasma $[Cr]_p$	Cell $[Cr]_c$	Ratio $\frac{[Cr]_c}{[Cr]_p}$	Plasma $[Urea\ N]_p$	Cell $[Urea\ N]_c$	Ratio $\frac{[Urea]_c}{[Urea]_p}$
minutes	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Before addition of urea and creatinine	0.022	0.021	0.95	0.256	0.202	0.79
Immediately after addition	0.330	0.045	0.13	0.708	0.554	0.78
30	0.305	0.057	0.19	0.710	0.554	0.78
60	0.295	0.087	0.30	0.715	0.541	0.76

20 per cent of its creatinine in perfusing the kidneys, the cell creatinine is not measurably changed.

Of urea, in contrast, about an equal proportion is removed by the kidneys from both cells and plasma. Presumably, urea is actually removed

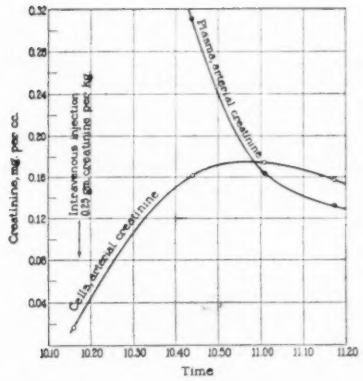


Fig. 1. The distribution of creatinine between plasma and cells in arterial blood after a single injection of 0.25 gram of creatinine per kilogram.

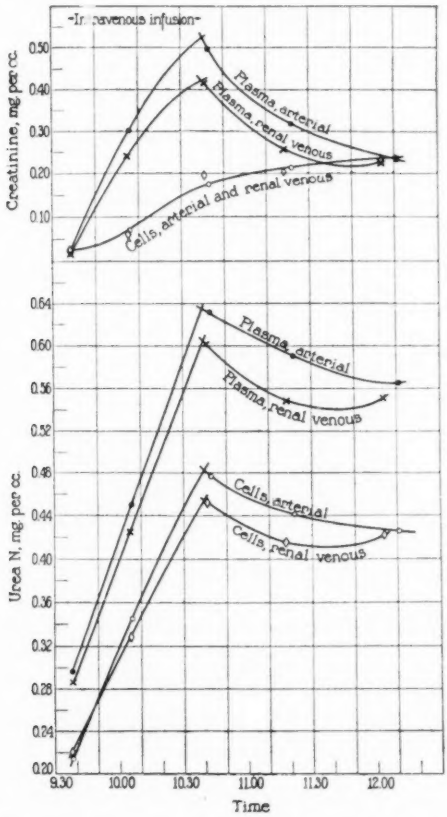


Fig. 2. The distribution of creatinine and urea between plasma and cells in arterial and renal venous blood during the course of an experiment on dog C3, during and after intravenous infusion for one hour with a solution containing 0.5 gram of urea and 0.25 gram of creatinine per kilogram.

●——● arterial plasma; ×——× renal venous plasma; ○——○ arterial cells; ◇——◇ renal venous cells.

from the blood only with the filtered plasma water, and decrease in cell urea concentration occurs after reabsorption of the filtered water; this dilutes the plasma urea, and causes diffusion of urea from the cells, until the concentration of urea per unit of water is the same in cells and plasma.

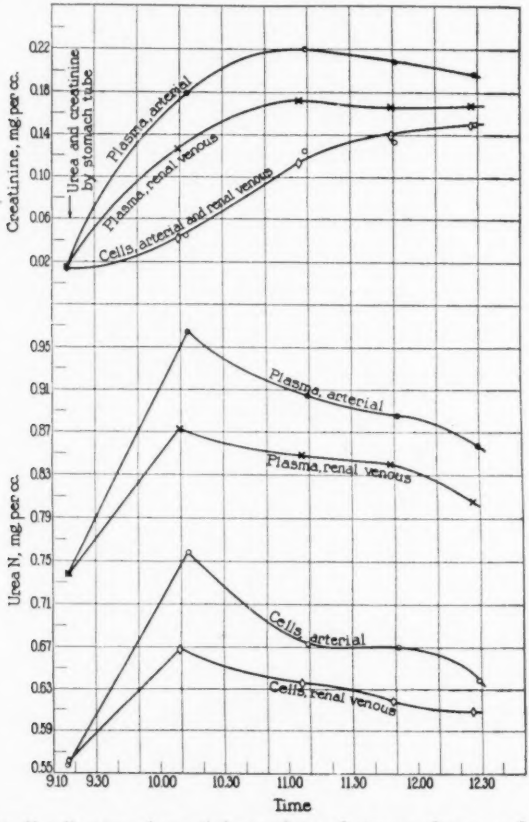


Fig. 3. The distribution of creatinine and urea between plasma and cells in arterial and renal venous blood during the course of an experiment on dog CO, after administration of 0.5 gram urea and 0.25 gram creatinine per kilogram, by stomach tube.

●——● arterial plasma; ×——× renal venous plasma; ○——○ arterial cells; ◇——◇ renal venous cells.

Whether the diffusion is complete when the renal vein blood is drawn, or occurs before and during centrifugation, is uncertain.

The equilibrium for creatinine is finally established after several hours, whereas for urea it is a matter of at most a few minutes, and perhaps of

seconds. One would expect from the laws of diffusion that the rates would be governed by the ratio of the squares of their molecular weights, that is, urea should enter the cells at about 3.5 times the speed of creatinine. The difference in rates of diffusion is much greater, and hence appears to be affected by factors other than the relative molecular dimensions.

DISCUSSION AND CALCULATIONS. *The effects of cell-plasma distributions of ferrocyanide, inulin, creatinine and urea on the relative plasma and whole blood extraction percentages.* The effects are calculated from the following observed quantitative relations: 1. Ferrocyanide and inulin do not penetrate the blood cells to an extent detectable by analysis (results in present paper).

2. Urea diffuses almost instantly to and from the blood cells, and is distributed through cells and plasma in the same concentration, per unit of water present (Peters and Van Slyke, 1931, p. 335–6). When the concentration in the plasma is altered, diffusion to or from the cells immediately restores the balance (present paper).

3. Creatinine is also distributed between cells and plasma. However, it diffuses so slowly that when it is partly removed from the plasma by the kidney, analysis of the immediately centrifuged cells of the renal blood shows no change in creatinine content from arterial cells. The decrease in the creatinine content of renal blood below the content of arterial blood is due entirely to decrease in the creatinine of the plasma (present paper).

Symbols:

E_p = percentage extraction of substances from plasma

$$= 100 \times \frac{\text{(conc. in arterial plasma)} - \text{(conc. in renal plasma)}}{\text{conc. in arterial plasma}}$$

E_B = percentage extraction of substances from whole blood, similarly calculated.

V_p = Volume of plasma in 1 volume of blood.

V_c = Volume of cells in 1 volume of blood.

$[H_2O]_p$ = grams H_2O in 1 cc. of plasma.

$[H_2O]_c$ = grams H_2O in 1 cc. of cells.

$[Cr]_p$, $[Cr]_c$, and $[Cr]_B$ = creatinine concentration, in milligrams per cubic centimeter, in plasma, cells, and whole blood.

Calculation of the ratio of whole blood to plasma extraction percentages: Ferrocyanide and inulin. Since both inulin and ferrocyanide are absent from the cells, the fraction of their plasma content extracted represents also the extracted fraction of their whole blood content. Hence:

$$E_B = E_p \tag{1}$$

for ferrocyanide and inulin.

Creatinine. The percentage extraction of creatinine from whole blood, however, is less than that from plasma, in proportion as the amount of creatinine in 1 cc. of arterial blood exceeds the amount in the plasma of 1 cc. of the blood. This conclusion follows from (3) of the "observed quantitative relations" stated above.

$$\frac{E_B}{E_p} = \frac{\text{Creatinine in plasma of 1 cc. of arterial blood}}{\text{Creatinine in whole of 1 cc. of arterial blood}} \tag{2}$$

We may substitute $V_p\ [\text{Cr}]_p$ for the creatinine in the plasma of 1 cc. of blood, and $[\text{Cr}]_B$ for the creatinine in 1 cc. of blood. We thus obtain

$$\frac{E_B}{E_p} = \frac{V_p\ [\text{Cr}]_{p\ \text{Arterial}}}{[\text{Cr}]_{B\ \text{Arterial}}} \tag{3}$$

Urea. The rapid diffusion of urea between cells and plasma causes the concentration in the cells to parallel that in the plasma, regardless of the amounts added to or withdrawn from the latter. Therefore the percentage by which urea concentration in the renal vein falls below that in the arteries is the same for plasma, cells, and whole blood. Hence equation 1 holds for urea as well as for ferrocyanide and inulin, although for quite a different reason.

Calculated relations of percentage extractions of ferrocyanide, inulin, creatinine and urea to the fraction of plasma water filtered. These calculations are based partly on the observed relationships between whole blood and plasma extractions developed in the preceding section, and partly on assumptions deduced from the filtration-reabsorption theory.

The assumptions based on the filtration-reabsorption theory are the following:

1. In the glomeruli a fraction, α, of the arterial plasma water is filtered out, together with an equal fraction of the filterable plasma solutes, which include the urea, creatinine, inulin, and ferrocyanide treated in our calculations. In the filtrate each such solute therefore has the same ratio, solute to water, as in the arterial plasma. That this ratio is maintained has been indicated, within the limits of analytical accuracy, for sugar (Walker and Reisinger, 1933), creatinine (Bordley, Hendrix and Richards, 1933), chloride (Wearn and Richards, 1925; Richards, Livingston and Freeman, 1930, and Westfall, Findley and Richards, 1934); uric acid (Bordley and Richards, 1933) and urea (Walker and Elsom, 1931) in the glomerular filtrate of frog's urine. Consequently filtration does not in itself change the concentration, per unit of water present, of any of the filterable constituents left in the plasma. In plasma of the blood in the efferent arterioles from the glomeruli the concentrations of these constituents are the same as in the plasma of arterial blood.

2. In the tubules the filtered water is reabsorbed into the blood with

such approximate completeness (usually over 97 per cent in dog or man) that the plasma volume is restored to practically its arterial size. The reabsorbed water therefore diminishes the concentration of each filterable solute in plasma of the renal vein blood to the fraction, $1 - \alpha$, of its concentration in arterial plasma, if none of the filtered solute is reabsorbed with the water.

3. Creatinine, inulin, and ferrocyanide are not reabsorbed at all with the water. Of the filtered urea, however, a fraction, R, is reabsorbed.

From the above observed facts and assumptions, the proportion of each of these four substances extracted from the whole blood and plasma can be formulated, as a function of the fraction of plasma water filtered, in the following manner.

Symbols (in addition to those of the preceding section):

α = fraction of arterial plasma water filtered in the glomeruli.

R = fraction of filtered urea which is reabsorbed in the tubules.

Ferrocyanide and inulin. The fraction of each filtered from the plasma is α, the same as the fraction of plasma water filtered. When the water is reabsorbed, without any ferrocyanide or inulin, the concentration of each in the plasma is reduced by the fraction α below its concentration in the arterial plasma. Since the percentage diminution in concentration represents by definition (see p. 635) the percentage extraction, the latter is equal to 100 α. Since (equation 1) for these two substances $E_B = E_p$, we have

$$E_B = E_p = 100\ \alpha \tag{4}$$

Creatinine. The filtration of plasma creatinine, and the dilution of the unfiltered portion by reabsorbed water, occur as outlined for ferrocyanide and inulin. Although the blood creatinine is partly in the cells, the cell creatinine diffuses out so slowly after dilution of the plasma by reabsorbed water that it does not measurably influence the concentration found in the renal vein plasma. Hence, as for inulin and ferrocyanide, the *plasma* extraction is

$$E_p = 100\ \alpha \tag{5}$$

for creatinine also.

To define E_B also in terms of α, we substitute for E_p in equation 3 its value, 100 α, from 5. Hence

$$E_B = 100\ \alpha\ \frac{V_p\ [\mathrm{Cr}]_{p\ \mathrm{Arterial}}}{[\mathrm{Cr}]_{B\ \mathrm{Arterial}}} \tag{6}$$

Urea. Urea excretion is similar to the process outlined for ferrocyanide, inulin, and creatinine, up to the moment when the filtered water is reabsorbed. Then two additional processes occur, each of which acts to

depress the percentage plasma extraction of urea below that of the above three substances.

1. Part of the filtered urea is reabsorbed with the water.

2. When the reabsorbed water diminishes the ratio of urea to water in the plasma, urea at once diffuses out of the cells to raise the plasma ratio until it meets that in the cells.

The two effects may be formulated respectively as follows:

1. If R represents the fraction of filtered urea which is reabsorbed, the amount of urea extracted from the blood by the kidneys is reduced to (1 − R) times the amount filtered. Thereby the urea extracted from 1 cc. of arterial blood is diminished from 100 α per cent of the amount in the *plasma* of 1 cc. of the blood to 100 α (1 − R) per cent of that amount.

2. In consequence of diffusion of urea from cells to plasma after water reabsorption, urea concentration in the renal venous plasma is lowered, not by 100 α (1 − R) per cent, but by this times the fraction $\frac{\text{H}_2\text{O in plasma of 1 cc. blood}}{\text{H}_2\text{O in whole of 1 cc. blood}}$. The diffusion also causes, as previously shown, the percentage decrease in urea concentration to be the same in cells and whole blood as in plasma. Hence, for urea,

$$E_B = E_p = 100\ \alpha(1 - R) \times \frac{\text{gram H}_2\text{O in plasma of 1 cc. blood}}{\text{gram H}_2\text{O in whole of 1 cc. blood}} \tag{7}$$

$$= 100\ \alpha(1 - R) \times \frac{V_p\,[\text{H}_2\text{O}]_p}{V_p\,[\text{H}_2\text{O}]_p + V_c\,[\text{H}_2\text{O}]_c} \tag{8}$$

$$= \frac{100\ \alpha(1 - R)}{1 + \dfrac{V_c\,[\text{H}_2\text{O}]_c}{V_p\,[\text{H}_2\text{O}]_p}} \tag{9}$$

From Van Slyke, Hastings, Murray and Sendroy (1925) one may take as approximate constants for normal blood of the horse, and probably with less than 10 per cent error for blood of dog or man, the values $[\text{H}_2\text{O}]_p = 0.93$ and $[\text{H}_2\text{O}]_c = 0.70$, hence the ratio $[\text{H}_2\text{O}]_c/[\text{H}_2\text{O}]_p = 0.75$. (This ratio may, however, vary several per cent even in the blood of the same animal.) Substituting 0.75 for the ratio in the last equation above gives, as *an approximation*

$$E_B = E_p = \frac{100\ \alpha(1 - R)}{1 + 0.75\,\dfrac{V_c}{V_p}} \tag{10}$$

As an example, representing conditions that may be encountered in a normal dog, take $V_c = 0.40$, $V_p = 0.60$, $\alpha = 0.20$, and $R = 0.40$. Filtration of 20 per cent of the plasma water and crystalloids, followed by reabsorption of the water, lowers the concentration of creatinine, inulin,

and ferrocyanide in the plasma of the renal vein to levels 20 per cent below those in arterial plasma, and gives a percentage extraction of 20 for each of these substances.

Of the filtered urea, however, 0.40 is reabsorbed with the water, so that the percentage extraction from the plasma is lowered, by reabsorption alone from 20 to 20 $(1 - 0.40) = 12$ for urea.

As the result of diffusion of urea from cells to plasma, fall in urea concentration is, however, distributed between cells and plasma, so that instead of being 12 per cent in the plasma it is $12 \times \frac{1}{1 + 0.75 \times \frac{0.4}{0.6}} = 8$ per cent in both cells and plasma (equation 10).

TABLE 4

Summary of calculated relations of percentage extractions to α, the fraction of plasma water filtered, and (in case of urea) to the fraction reabsorbed

SUBSTANCE	PLASMA PERCENTAGE EXTRACTION, E_p	WHOLE BLOOD PERCENTAGE EXTRACTION, E_B
Ferrocyanide	100α	Same as E_p
Inulin	100α	Same as E_p
Urea	$\frac{100\alpha(1 - R)}{1 + \frac{[H_2O]_c V_c}{[H_2O]_p V_p}}$	Same as E_p
Creatinine	100α	$\frac{100\alpha V_p [Cr]_{p\ \text{Arterial}}}{[Cr]_{B\ \text{Arterial}}}$

R = fraction of filtered urea which is reabsorbed.

$[H_2O]_c$ and $[H_2O]_p$ = grams water per cubic centimeter cells and plasma.

V_c and V_p = volumes of cells and plasma in 1 volume whole blood.

$[Cr]_p$ and $[Cr]_B$ = milligrams creatinine per cubic centimeter plasma and whole blood.

It will be noted that, although only 40 per cent of the filtered urea is reabsorbed, the added effect of diffusion from cells to plasma lowers the percentage plasma urea extraction to 40 per cent, instead of 60 per cent, of the creatinine, inulin, or ferrocyanide extraction.

Calculation, from urea extraction percentage, of proportion of filtered urea which is reabsorbed. By solving equation 10 for R, the fraction reabsorbed, we obtain, for urea,

$$R = 1 - \frac{E_p}{100\,\alpha}\left(1 + 0.75\,\frac{V_c}{V_p}\right) \tag{11}$$

(E_B may be substituted for E_p, since both are equal.)

From data in the preceding paper we take the following as approximate mean values, $E_p = 8$, and $\alpha = 0.20$; α being calculated by equation 1

or 2 from a mean ferrocyanide, inulin, or creatinine percentage extraction of 20. The average hematocrit reading in our experiments was 0.42, so that

$$V_c = 0.42 \text{ and } V_p = 0.58$$

We calculate from these figures the approximate mean value of R as

$$R = 1 - \frac{8}{20}\left(1 + 0.75\,\frac{0.42}{0.58}\right) = 0.38$$

This is in reasonably good agreement with the value, 0.43, calculated in the preceding paper, from the mean ratio of the *clearance* of urea to the clearances of ferrocyanide, creatinine, and inulin.

A summary of the various calculations is given in table 4.

SUMMARY

1. Sodium ferrocyanide and inulin injected intravenously into a dog circulate in the plasma without entering the erythrocytes in measurable amounts.

2. Creatinine absorbed or injected into the circulation gradually enters the blood cells. However, diffusion between cells and plasma is so slow that, when the blood perfuses the kidneys, where plasma creatinine concentration falls 20 per cent, the fall in cell creatinine is too slight to measure.

3. Urea diffuses so quickly that when added to blood it reaches equilibrium between cells and plasma before they can be separated for analysis. The rapid diffusibility also causes the observed percentage fall in urea concentration from arterial to renal venous blood to be the same in cells as in plasma.

4. The effects of these diffusibility differences on the relative concentration decreases of these substances in plasma and in whole blood during perfusion of the kidneys have been calculated.

5. From comparison of the mean plasma extraction percentage of urea with that of the presumably non-reabsorbed substances, ferrocyanide, inulin and creatinine, it is calculated that in the dog an average of 38 per cent of the filtered urea is reabsorbed. This figure agrees reasonably well with the value, 43 per cent, obtained in the preceding paper from comparison of clearances.

REFERENCES

BERGLUND, H. J. A. M. A. **79:** 1375, 1922.

BORDLEY, J., 3RD, J. P. HENDRIX AND A. N. RICHARDS. J. Biol. Chem. **101:** 255, 1933.

BORDLEY, J., 3RD AND A. N. RICHARDS. J. Biol. Chem. **101:** 193, 1933.

FOLIN, O. AND H. BERGLUND. J. Biol. Chem. **51:** 395, 1922.

GAD-ANDRESEN, K. L. Biochem. Ztschr. **116:** 266, 1921.

HUNTER, A. AND W. R. CAMPBELL. J. Biol. Chem. **32:** 195, 1917.

PETERS, J. P. AND D. D. VAN SLYKE. Quantitative clinical chemistry. Vol. I, 1931. The Williams and Wilkins Co., Baltimore.
RHOADS, C. P. Science **73:** 417, 1931.
This Journal **109:** 324, 1934.
RICHARDS, A. N., A. E. LIVINGSTON AND B. FREEMAN. J. Biol. Chem. **87:** 467, 1930.
VAN SLYKE, D. D., A. B. HASTINGS, C. D. MURRAY AND J. SENDROY, JR. J. Biol. Chem. **65:** 701, 1925.
VAN SLYKE, D. D., A. HILLER AND B. F. MILLER. This Journal, **113:** 611, 1935.
WALKER, A. M. AND K. A. ELSOM. J. Biol. Chem. **91:** 593, 1931.
WALKER, A. M. AND J. A. REISINGER. J. Biol. Chem. **101:** 223, 1933.
WEARN, J. T. AND A. N. RICHARDS. J. Biol. Chem. **66:** 247, 1925.
WESTFALL, B. B., T. FINDLEY AND A. N. RICHARDS. J. Biol. Chem. **107:** 661, 1934.
WILSON, D. W. AND E. D. PLASS. J. Biol. Chem. **29:** 413, 1917.
WU, H. J. Biol. Chem. **51:** 21, 1922.

A COMPARISON OF THE ANEMIA PRODUCED BY FEEDING YOUNG RATS UPON HUMAN, COW AND GOAT MILK

HOWARD H. BEARD AND THOMAS S. BOGGESS

From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans

Received for publication May 16, 1935

Numerous workers have produced nutritional anemia in young rats by the exclusive feeding of cow's milk. Much work has also been done in this connection using goat's milk. Bomskov and co-workers (1, 2, 3, 4) observed that the exclusive feeding of cow's milk to 4 and 8 week old rats led to hypochromic anemia characteristic of Fe deficiency which was cured by the administration of Fe. When their 8 week old animals were fed goat's milk, the same anemia was produced as that obtained by feeding cow's milk. In the 4 week old animals, however, a hyperchromic anemia, which was not cured by Fe, was produced by feeding the goat's milk. György (5) and other clinicians have also observed a hyperchromic anemia in infants by feeding on goat's milk. Fe, or Fe and Cu, or lactoflavin (vitamin B_2) were ineffective, while liver therapy was very quickly effective in the cure of this anemia. György and Rominger and co-workers stated that the anemia was due to a lack of Castle's extrinsic factor, sometimes accompanied by a lack of Fe.

Schiff, Eliasberg and Joffe (6) were the only workers who have used human milk in nutritional anemia studies. The interesting finding was made that human milk will not produce the characteristic anemia found when cow's milk is fed and lactose was stated to be the protective agent.

During the past year we have made a study of the different types of anemia that may be produced by the exclusive feeding of cow, goat and human milk the results of which are given below.

EXPERIMENTAL. The technique used in all studies has been described in our original publications (7). The young animals never had access to their mother's diet, and at 23 days of age were separated into individual cages and fed exclusively on human milk (obtained from wet nurses at Charity Hospital), goat's milk (obtained from 2 goats kept upon the hospital grounds and milked into glass containers), or cow's milk (which was obtained from the daily hospital supply). A total of 9 litters was used and the animals were divided as follows: 17 on human milk, 28 on cow milk and 33 on goat milk. The determinations of the body weight, hemo-

globin content and erythrocyte count were made at weekly intervals. Ten young animals killed by slow starvation and vitamin deficiencies and 10 adult rats fed with cow's and goat's milk served as controls for the experimental animals.

Stained blood smears were studied for the morphological blood picture and the presence of Bartonella muris. As soon as an animal died an autopsy was performed and the tissues were fixed in formalin for microscopic study. Bone marrow smears were stained with Giemsa solution for the differential count of the cells.

TABLE 1

The effect of Fe, with and without Cu, upon the prevention of goat milk anemia (duration of experiment six weeks)

NUMBER OF ANIMALS AND DIET	BODY WEIGHT (GRAMS)						HEMOGLOBIN PER 100 CC. (GRAMS)						ERYTHROCYTES PER C.MM. (MILLIONS)					
	Before			After			Before			After			Before			After		
	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average
9 Goat milk	29	40	**30**	57	80	**66**	8.4	11.9	**9.8**	4.0	7.0	**4.9**	3.6	4.9	**4.2**	2.2	4.5	**3.2**
19 Goat milk plus 0.25 mgm. Fe daily	25	46	**33**	66	115	**90**	8.1	15.5	**10.8**	5.7	16.8	**11.7**	3.5	4.9	**4.1**	4.1	7.8	**6.0**
20 Goat milk plus 0.25 mgm. Fe plus 0.05 mgm. Cu daily	24	50	**34**	74	109	**87**	9.1	17.4	**11.5**	10.5	20.2	**15.0**	3.6	4.6	**4.1**	5.1	7.9	**6.2**

The effect of Fe, with and without Cu, upon prevention of the goat milk anemia was also studied. We used 9 animals on goat milk, 19 on goat milk with Fe, and 20 on goat milk with Fe and Cu.

RESULTS. The average results obtained are shown in the charts and table 1. In chart 1 it is seen that the feeding of human milk was slightly better than cow's milk on growth, while the poorest growth was obtained with goat's milk. In chart 2 the feeding of human milk resulted in the maintenance of the average Hb level of about 9.5 to 11 grams per 100 cc. The Hb dropped more quickly on goat's milk than it did on cow's milk. In chart 3 it is seen that the average red cell count of 5.2 m. per c.mm. increased to 8.6 m. per c.mm. in 11 weeks in the animals fed upon human

milk, while the drop in cells was much quicker the first two weeks in the animals fed goat's milk as compared with that of those fed on cow's milk. This fact explains the hyperchromic character of the goat's milk anemia during the early part of the experiment. In no case, however, did the color index go above 1, which is the typical value in human pernicious anemia. The red blood picture showed in most of the animals no difference according to diet.

A study of the blood smears by Doctor von Haam (8) showed marked anisocytosis and poikilocytosis with polychromasia of the erythrocytes. The number of reticulocytes was considerably higher in the animals fed goat's milk. Nucleated red blood cells could occasionally be found in all

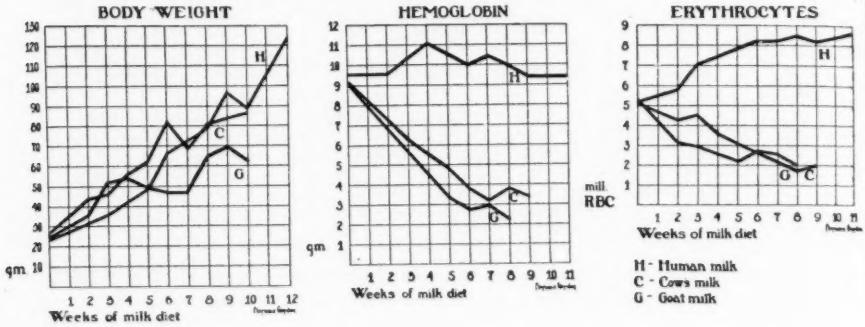


Chart 1 Chart 2 Chart 3

Chart 1. Showing the effect of feeding human, cow and goat milk upon growth of young rats.

Chart 2. Showing the effect of feeding human, cow and goat milk upon the hemoglobin content of young rats.

Chart 3. Showing the effect of feeding human, cow and goat milk upon the erythrocytes of young rats.

anemic animals while megaloblasts were missing. In none of the animals, however, was the red blood picture as strikingly different as that described by Rominger (1, 4). There was no principal difference between the pathological changes occurring in goat's milk and cow's milk anemia except that the damage to the bone marrow was more severe in goat's milk anemia. Letterer's (9) observation concerning the disappearance of mature white blood cells from the bone marrow during goat's milk anemia could be confirmed to a certain degree.

The gross autopsy findings of the anemic animals were so identical that no difference could be made macroscopically between the post mortem picture of goat's milk and cow's milk anemia. The degree of anemia seemed to be the only influential factor. Marked hypertrophy, as previously

observed by Daniels and Burright (10) and dilatation of the heart, were observed in all instances. Those animals killed in the early stages of the anemia showed only hypertrophy, while the dilatation was observed in those animals which died spontaneously with a red cell count below 2 m. per c.mm. The lungs showed a varying degree of emphysema. The spleen was enlarged in some cases but not in others. The intestines, kidneys and endocrine glands showed nothing except the anemia.

One of the most characteristic gross pathological findings in the anemic animals was the appearance of the liver. There was fatty degeneration and the organ was usually smaller than normal with a fine granular surface. Its color was pale yellow and the cut surface showed a whitish-yellow mottling. The control animals showed none of these findings which could be considered as characteristic of nutritional anemia of the rat.

Anemia was produced in all rats fed upon goat milk. Four animals on Fe developed a slight anemia, but the average Hb and red cell counts on Fe, with and without Cu, were maintained, Cu having a slightly better effect on the average rise in Hb than Fe alone.

Discussion. We have confirmed the finding of Schiff and co-workers (6) that human milk will not produce the characteristic anemia found in young rats fed upon either goat or cow milk. Since most of the animals fed on either goat or cow milk alone died of anemia, it is evident that the lactose content of these milks did not prevent the onset of the anemia. This fact makes it difficult to understand why the lactose in human milk can prevent and cure milk anemia, as stated by Schiff. Since our animals fed upon human milk made somewhat better growth than those on the other milks, it is possible that the milk intake may have played a part in the prevention of the anemia in this case. A review of the literature by Davidson and Leitch (11) shows that the average Fe content of the three types of milk used in this study is about the same. Shohl (12), however, stated that human milk contains about 3 or 4 times as much Fe as cow's milk. Davidson and Leitch also stated that human milk contains about 4 times as much Cu as cow's milk. It is possible, therefore, that the higher Fe and Cu content of the human milk were the protective factors against the onset of anemia.

The Fe and Cu content of cow and goat milk is about the same (11). We (7) have previously shown that Fe alone will prevent and cure the cow milk anemia, and since Fe, with or without Cu, prevented the onset of the goat milk anemia, we believe that the feeding of goat's milk produces a hypochromic type of anemia characteristic of Fe deficiency.

The results of this study also demonstrate the profound changes that take place in the young animal body when insufficient amounts of Fe are ingested. In addition to preventing the anemic changes in the experimental animals, Fe can also prevent the development of cardiac hyper-

trophy, atrophy of the spleen and fatty degeneration of the liver. These findings emphasize anew the great importance of Fe in the physiological economy of the animal body.

SUMMARY

The anemia produced by feeding young weanling rats upon human, goat's and cow's milk was compared. The following results were obtained:

1. The feeding of human milk did not produce anemia.
2. The drop in erythrocytes and hemoglobin in the cases of cow's milk anemia was somewhat slower than in the cases of goat's milk anemia.
3. Hypertrophy of the heart muscle, atrophy of the spleen, and fatty degeneration of the liver, were the most consistent gross pathological findings in the anemic rats. The importance of iron in preventing these changes was discussed.
4. The pathological findings in experimental goat's milk anemia are not similar to those in pernicious anemia of man, and any close relationship between both types of anemia must be denied.
5. Iron, with and without copper, prevented the onset of the goat's milk anemia.

We wish to express our thanks to the American Academy of Arts and Sciences for a grant from their Permanent Science Fund for these studies.

REFERENCES

(1) Rominger, E., H. Meyer and C. Bomskov. Ztschr. f. d. ges. exp. Med. **89**: 786, 1933.
(2) Bomskov, C. and M. Auffarth. Ztschr. f. d. ges. exp. Med. **89**: 804, 1933.
(3) Bomskov, C. and H. Czerlinsky. Ztschr. f. d. ges. exp. Med. **89**: 809, 1933.
(4) Rominger, E. and C. Bomskov. Ztschr. f. d. ges. exp. Med. **89**: 818, 1933.
(5) György, P. Ztschr. f. Kinderh. **56**: 1, 1934.
(6) Schiff, E., H. Eliasberg and N. Joffe. Jahrb. f. Kinderh. **139**: 143, 1933.
(7) Beard, H. H. and V. C. Myers. J. Biol. Chem. **94**: 71, 1931.
(8) von Haam, E. Southern Med. J. **28**: 22, 1935.
(9) Letterer, E. Jahrb. f. Kinderh. **130**: 1, 1930.
(10) Daniels, A. L. and I. Burright. Proc. Soc. Exp. Biol. and Med. **30**: 857, 1933.
(11) Davidson, L. S. P. and I. Leitch. Nutr. Abst. and Rev. **3**: 901, 1934.
(12) Shohl, A. T. Textbook of biochemistry (Harrow and Sherwin), W. B. Saunders Co., Philadelphia and London, 1935, p. 651.

FURTHER OBSERVATIONS UPON THE ORIGIN OF CREATINE FROM PROTEINS AND AMINO ACIDS

HOWARD H. BEARD AND THOMAS S. BOGGESS

From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans

Received for publication July 15, 1934

Several reviews of the literature on creatine and creatinine metabolism since the appearance of Hunter's monograph (1) have been published by Mitchell and Hamilton (2), Myers (3), Rose (4) (5) and Kayser (6) (7). According to Kayser recent evidence shows that arginine, histidine, purines, betaine, choline and amino acids are to be considered as possible precursors of creatine. Experimental and clinical researches have definitely established the relation of glycine to creatine-creatinine metabolism. Kayser believes that the transformation of glycine into creatine is direct, a view also held by Thomas, Milhorat and Techner (8), Kostakow and Slauck (9), Nevin (10) and Beard and Barnes (11). It is our belief that experimental and clinical researches of the future dealing with amino acid therapy in several pathological conditions will continue to show that creatine is a product of the exogenous protein or amino acid metabolism, although this view is at present not accepted by Rose (4) (5), Chanutin (12) Bodansky (13), and others.

It is well known that it is difficult to increase the creatine concentration of normal muscle tissue by protein and amino acid feeding. This difficulty can, however, be overcome if large quantities of these substances are fed (11). But if this is done the criticism (4) (6) has been made that these large quantities of amino acids were possibly toxic. Nevertheless we found no pathological changes in any of our experimental animals, a report being published on the kidneys (14). Several investigators (15) have shown that the renal inflammation sometimes observed is variously affected by the manner of administration and the relationship of the amino acid to the other foodstuffs given at the same time. It is generally believed that glycine or glutamic acid are non-toxic when given in 10 to 30 gram daily doses to patients suffering from various myopathies. We wish to state again that if large quantities of creatine precursors are not fed then no increases in the creatine content of the muscles are to be observed.

Most of our myopathy patients (16) (17) when questioned, stated that

they had eaten very little meat, fish, or eggs for some time, either due to a dislike for these foods or to economic reasons. Reinhold and co-workers (18) stated that in some of their advanced cases of muscular dystrophy only 5 to 10 per cent of the normal nitrogen and creatine contents of the muscles were to be found. The creatine content of the muscles of some patients after glycine therapy was increased to three or four times their original values. In this connection Professor Rose (5) has stated "Perhaps a more satisfactory approach to the problem of the origin of an anabolic substance would be to limit the intake of a suspected precursor below the required level, rather than to add excessive quantities to a diet already carrying sufficient amounts to meet the demands of synthesis."

With these ideas in mind we have attempted to limit the intake of creatine precursors (proteins and amino acids) in the diet of normal adult rats and then refeed them with a liberal intake of same. In this way we may observe the changes in some of the chemical constituents of the muscle tissue. The results obtained are given below.

EXPERIMENTAL. Normal adult rats weighing between 175 and 270 grams were used and fed upon the following diets (table 1).

The commercial egg albumin was coagulated, washed and dried. The modified Karr salt mixture was composed of the following ingredients NaCl, 10; $CaCO_3$ 1.3; MgO, 1.25; $FeCl_3$, 1.09. The bone ash was prepared by ashing bone black.

The animals were killed by a blow on the back of the head. Creatine (total creatinine) was determined upon 1 gram samples of muscle tissue by the method of Rose, Helmer and Chanutin (19) with the usual precautions. Two gram samples of muscle tissue were dried to constant weight at 105°C. for total solids. The dried residues were then extracted for 18 hours in Soxhlet extractors with anhydrous ether for the fat content. The total nitrogen on these moisture and fat-free residues was then determined by the macro-Kjeldahl method.

RESULTS. The results obtained on the fresh muscle tissue are given in table 2 (egg albumin) and table 3 (casein).

Total nitrogen and total solids. The changes observed in these constituents are considered insignificant.

Ether extract. Very few changes were noted in the fat content of the muscles in the egg albumin experiments. Some slight increase in fat was observed on the 4 per cent casein diet (group H) over the protein-free diet (group A) and on the 21 per cent glutamic acid diet (group K) over the 4 per cent casein diet (group H). Little significance is attached to these increases due to the wide variations in fat content of any of the groups of animals.

Total creatine. As was to be expected the most significant increases in this constituent occurred after refeeding either glycine, glutamic acid,

TABLE 1

Diet

	CONTROL A	PROTEIN B	GLYCINE C	GLUTAMIC ACID D	GLYCINE I
Egg albumin	4	25	4	4	4
Glycine			21		10
Glutamic acid				21	
Butter fat	5	5	5	5	5
Lard	15	15	15	15	15
Starch	53	32	32	32	43
Sucrose	18	18	18	18	18
Salt mixture (modified Karr)	2	2	2	2	2
Bone ash	3	3	3	3	3

	CONTROL E	PROTEIN F	GLYCINE G	GLUTAMIC ACID H	PROTEIN-FREE DIET
Casein	4	25	4	4	
Glycine			21		
Glutamic acid				21	
Butter fat	5	5	5	5	5
Lard	15	15	15	15	15
Starch	53	32	32	32	57
Sucrose	18	18	18	18	18
Salt mixture (modified Karr)	2	2	2	2	2
Bone ash	3	3	3	3	3

The stock diet was Sherman's diet B—⅔ whole wheat flour, ⅓ whole milk powder, with 1 per cent of the weight of the wheat, each, as NaCl and $CaCO_3$. Dried yeast, 200 mgm., tablet daily, was fed to all animals.

The distribution of the animals was as follows:

GROUP	LENGTH OF TIME FED ON DIET
A	9 weeks on protein-free experimental diet
B	9 weeks on stock diet (Sherman's diet B)
C	A for 9 weeks
D	A for 5 weeks then diet B for 4 weeks
E	A for 5 weeks then diet I for 4 weeks
F	A for 5 weeks then diet C for 4 weeks
G	A for 5 weeks then diet D for 4 weeks
H	E for 9 weeks
I	E for 5 weeks then diet F for 4 weeks
J	E for 5 weeks then diet G for 4 weeks
K	E for 5 weeks then diet H for 4 weeks

egg albumin or casein, over their respective control groups fed on either 4 per cent egg albumin (group C) or 4 per cent casein diets (group H), with one exception. Refeeding group E on 10 per cent glycine after 5 weeks on 4 per cent egg albumin gave only a slight increase in the creatine content over the control (group C) on the 4 per cent egg albumin diet.

TABLE 2

Effect of refeeding egg albumin, glycine or glutamic acid with the 4 per cent egg albumin diet upon the chemical composition of muscle

GROUP	NO. OF ANIMALS	DIET	TOTAL NITROGEN			ETHER EXTRACT			TOTAL CREATINE			TOTAL SOLIDS			BODY WEIGHT	
			Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Before	After
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*gms.*	*gms.*
A	5	No protein	3.97	2.76	3.64	2.30	1.47	1.87	0.50	0.39	0.44	25.56	25.09	25.45	177	156
B	10	Stock	3.96	3.39	3.69	2.14	0.67	1.53	0.36	0.32	0.35	25.06	26.80	25.58	217	228
% increase...		Group B over A			None			None			None			None		
C	15	(A) 4% egg albumin	4.07	3.60	3.82	4.11	1.98	2.63	0.67	0.51	0.59	28.12	24.44	25.93	189	159
% increase...		Group C over A			4.9			40.6			34.1			None		
D	15	(B) 25% egg albumin	4.38	3.79	4.14	3.60	2.07	2.97	0.84	0.68	0.71	27.44	25.60	26.45	179	177
% increase...		Group D over C			8.3			12.9			20.3			2.0		
E	5	4% egg albumin (I) +10% glycine	3.91	3.60	3.76	2.00	1.14	1.68	0.83	0.55	0.63	27.23	25.40	26.01	221	197
% increase...		Group E over C			None			None			6.8			None		
F	10	4% egg albumin (C) +21% glycine	3.97	2.76	3.63	2.48	0.60	1.33	1.05	0.81	0.87	26.08	24.44	25.10	218	159
% increase...		Group F over C			None			None			47.4			None		
G	10	4% egg albumin (D) +21% glutamic acid	4.90	2.23	3.64	1.21	0.62	0.96	0.96	0.68	0.81	25.89	24.06	24.79	229	164
% increase...		Group G over C			None			None			37.3			None		

An average increase of 20 per cent in muscle creatine was obtained in the animals fed upon the 25 per cent egg albumin diet (group D) as compared to 4 per cent of this protein (group C). When either 21 per cent of glycine (group F) or glutamic acid (group G) was added to the 4 per cent egg albumin diet (group C) increases of 47 and 37 per cent respectively in muscle creatine were obtained. Refeeding 25 per cent casein (group I)

to the 4 per cent casein diet (group H) caused an increase in muscle creatine of about 54 per cent. The same increases occurred by refeeding 21 per cent glycine (group J) and 21 per cent glutamic acid (group K).

Groups A (no protein) and B (stock diet) are included in both tables for comparison. These groups are, however, not strictly comparable with the experimental groups D to K due to the different diets fed. Nevertheless it is evident that the average concentration of creatine in the experimental groups D to K are much higher in comparison with groups A and B, than they are with the control groups C and H (4 per cent egg

TABLE 3

Effect of refeeding casein, glycine or glutamic acid with the 4 per cent casein diet upon the chemical composition of muscle

GROUP	NO. OF ANIMALS	DIET	TOTAL NITROGEN			ETHER EXTRACT			TOTAL CREATINE			TOTAL SOLIDS			BODY WEIGHT	
			Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Maximum	Minimum	Average	Before	After
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*gms.*	*gms.*
A	5	No protein	3.97	2.76	3.64	2.30	1.47	1.87	0.50	0.39	0.44	25.56	25.09	25.45	177	156
B	10	Stock	3.96	3.39	3.69	2.14	0.67	1.53	0.36	0.32	0.35	25.06	26.80	25.58	217	228
H	15	(E) 4% casein	4.08	3.03	3.73	3.00	1.96	2.43	0.43	0.36	0.39	28.69	26.79	27.44	234	175
% increase...		Group H over A			2.4			30.0			None			7.8		
I	15	(F) 25% casein	3.89	3.69	3.71	2.83	1.41	2.09	0.70	0.50	0.60	28.70	22.98	26.39	232	172
% increase...		Group I over H			None			None			53.8			None		
J	10	(G) 4% casein + 21% glycine	3.94	3.62	3.81	3.69	1.77	2.46	0.64	0.57	0.61	29.13	24.52	26.43	267	196
% increase...		Group J over H			2.1			None			56.4			None		
K	10	(H) 4% casein + 21% glutamic acid	4.07	3.78	3.93	4.19	2.47	3.23	0.65	0.53	0.60	31.17	28.00	30.02	223	163
% increase...		Group K over H			5.3			32.9			53.8			9.4		

albumin and 4 per cent casein). These large increases over the protein-free and stock diets will not seem so impossible when it is recalled that there are variations from 40 to 162 per cent in muscle creatine under normal conditions. Thus the significance of these increases cannot be evaluated at this time.

No pathological changes were observed in the organs of any animals used in this study.

Discussion. These experiments were planned to more or less simulate the conditions that we find in patients suffering from various myopathies. A low protein, rather than a protein-free diet, was selected. The protein-

free group A was run to see if the partial protein starvation would cause an increase in muscle creatine. But the reverse was true, slightly less creatine being found in complete protein starvation than when the animals were fed upon the 4 per cent protein diets. It is generally believed by some investigators that complete starvation causes an increase in muscle creatine but this is denied by others. The increases in creatine obtained in the experimental groups D to K cannot be due to the partial protein starvation for the first 5 weeks of the test. The increases in creatine in the experimental groups are also not due to differences in the water, fat or total nitrogen content of muscles.

We attach more significance to the fact, as shown by the data given in tables 2 and 3, that the *minimum concentration* of muscle creatine after refeeding on casein, egg albumin, glycine or glutamic acid is, in every case, the same or higher than the *maximum concentration* of creatine observed on the 4 per cent casein or egg albumin control groups.

Since we observed much larger increases in creatine formation from proteins and amino acids in the present experiments than formerly (11), we believe that protein starvation for 5 weeks before the possible precursors are fed is a much better experimental procedure for studying the origin of creatine than using normal well-fed animals. While the relation of glycine to creatine formation is established most workers failed to find that glutamic acid would give increases in muscle creatine or creatinine elimination. This amino acid in our hands has always given substantial increases in muscle creatine and creatine excretion and beneficial results in some of our myopathy patients (16) (17).

The large amount of evidence for the origin of creatine from exogenous precursors during the past 8 years shows that this substance can no longer be considered entirely as a product of the endogenous protein metabolism. When glycine causes an increase up to 50 per cent in muscle creatine and 1000 per cent in creatine excretion in the myopathies it is clearly seen that creatine metabolism in the organism is unstable and may be easily affected by many factors (proteins, amino acids, nuclear material, various hormones, etc.) in normal animals and in patients suffering from various pathological conditions. The endogenous formation of creatine from tissue proteins during starvation evidently occurs, but the ingestion of proteins and amino acids then causes an increased exogenous formation of creatine.

SUMMARY

Normal adult rats were fed on complete synthetic diets containing either 4 per cent casein or egg albumin as the sole protein for a period of 5 weeks. Refeeding these animals on 25 per cent of casein, or egg albumin, or 21 per cent of glycine or glutamic acid, for a period of 4 more weeks, caused

average increases in the total creatine of the muscles varying from 20 to 56 per cent above the control animals on the 4 per cent protein diets.

Total nitrogen, fat and total solid content of the muscles of all animals showed very little change.

Creatine is again shown to be a product of the exogenous catabolism of proteins and amino acids.

REFERENCES

(1) HUNTER, A. Creatine and creatinine. Longmans Green & Co., p. 75–78, 1928.
(2) MITCHELL, H. H. AND T. S. HAMILTON. The biochemistry of the amino acids. A. C. S. Monograph no. 48, p. 328, 1929.
(3) MYERS, V. C. Yale J. Biol. and Med. **4**: 467, 1932.
(4) ROSE, W. C. Ann. Rev. Biochem. **2**: 187, 1933.
(5) ROSE, W. C. Ann. Rev. Biochem. **4**: 243, 1935.
(6) KAYSER, F. Métabolismes des Corps Créatiniques. Actualités Scientifique et Industrielles. No. 179, Hermann et Cie, Éditeurs, Paris, p. 24–32, 1934.
(7) KAYSER, F. Créatine et Créatinine, Actualités Scientifiques et Industrielles. No. 178, Hermann et Cie, Éditeurs, Paris, p. 54–55, 1934.
(8) THOMAS, K., A. T. MILHORAT AND F. TECHNER. Ztschr. f. physiol. Chem. **205**: 93, 1932.
(9) KOSTAKOW, S. AND A. SLAUCK. Deutsch. Arch. Clin. Med. **175**: 302, 1933.
(10) NEVIN, S. Brain **57**: 239, 1934.
(11) BEARD, H. H. AND B. O. BARNES. J. Biol. Chem. **94**: 49, 1931.
(12) CHANUTIN, A. J. Biol. Chem. **89**: 765, 1930.
(13) BODANSKY, M. Proc. Am. Soc. Biol. Chem., J. Biol. Chem. **109**: xi, 1935.
(14) BEARD, H. H. AND R. A. MOORE. Proc. Soc. Exp. Biol. and Med. **29**: 30, 1931.
(15) KOTAKE, Y. Ann. Rev. Biochem. **4**: 225, 1935.
(16) TRIPOLI, C. J. AND H. H. BEARD. Arch. Int. Med. **53**: 435, 1934.
(17) TRIPOLI, C. J., W. M. MCCORD AND H. H. BEARD. J. A. M. A. **103**: 55, 1934.
(18) REINHOLD, J. G., J. H. CLARK, G. R. KINGSLEY, R. H. CUSTER AND J. W. MCCONNELL. J. A. M. A. **102**: 261, 1934.
(19) ROSE, W. C., O. M. HELMER AND A. CHANUTIN. J. Biol. Chem. **75**: 543, 1927.

THE INFLUENCE OF FREQUENCY OF CONTRACTION OF THE ISOLATED MAMMALIAN HEART UPON THE CONSUMPTION OF OXYGEN

A. E. COHN AND J. MURRAY STEELE

From the Hospital of the Rockefeller Institute for Medical Research, New York

Received for publication July 12, 1935

In interpreting a series of observations on Knowlton and Starling's (1) modification of Martin's (2) heart lung preparation, it appeared necessary to estimate as accurately as possible the effect of rate of cardiac contraction on the consumption of oxygen. Previous analyses of this relation are unsatisfactory for two reasons: first, the means used to alter cardiac rate were themselves capable of influencing the rate of consumption of oxygen, and second, the number of observations was insufficient for making a satisfactory analysis.

Evans' (3) (4) observations led him to believe that with change in cardiac rate oxygen consumption per beat remained constant except at very high or very low rates. To secure wide variations in rate, the temperature of his preparations was altered. But under these circumstances the effect of rate alone is obscured since the effect on metabolism may have been due to increase in speed of the chemical reactions alone due to van't Hoff's law.

Starling and Visscher (5) have since (1926) succeeded in altering the cardiac rate by changing vagal tone. By keeping the output constant, gross change in diastolic volume was prevented. It seems clear from their experiments that consumption of oxygen *per unit of time* varies *directly* but that consumption *per beat* varies *inversely* with frequency of contraction. The resultant effect of these interrelations is that when the rate doubles, for example, the amount of oxygen burned in unit time is somewhat less than doubled, due to the fact that each beat consumes less oxygen at the higher rate. It is far from certain however whether vagal stimulation may not itself alter the metabolism of the heart muscle either directly or through its influence on tone and contraction. In two of their experiments, when the diastolic volume was kept constant "by adjusting the output," the ratio of rate of change in oxygen consumption to rate of change in frequency of beat was approximately 0.5 or less (cf. 5, table 111, p. 259). But when under vagal stimulation the heart dilated, the output being kept constant, this ratio became 0.9 to 1.0 except in

the first observation (table 4, p. 259) $\frac{(5)}{(6)}$. In the presence of such a ratio the consumption of oxygen per beat remains of necessity unaffected even though the heart dilates, a result clearly contrary to Starling's law. The use of vagal stimulation to slow the cardiac rate appears, therefore, a questionable procedure.

TABLE 1

Changes in rate of consumption of oxygen with changes in frequency of contraction induced by electrical stimuli

DOG NO.	BEFORE STIMULATION		DURING STIMULATION		AFTER STIMULATION		CHANGE OF O_2 / CHANGE OF RATE
	Pulse	O_2	Pulse	O_2	Pulse	O_2	
1	162	4.23	162	4.28	143	4.29	0
5	151	3.46	153	3.50			0
2	127	3.42	188	4.80			0.69
	(Pericardium opened)		188	5.16	128	4.25	
9	156	4.40	185	4.89	148	4.11	0.67
10	130	5.15	147	5.35	108	4.22	0.63
11	159	4.80	189	5.36	149	4.66	0.58
12			167	5.91	140	5.40	0.40
13	142	5.29	188	6.39	135	5.44	0.55
14	134	5.04	170	5.28	138	5.42	0.54
3			158	4.88	136	4.32	0.69
4	138	4.16	151	4.60	130	4.36	0.75
15	122	4.52	158	5.78	128	4.81	0.89
16	128	4.20	160	4.88			0.64
17	146	5.40	180	5.93	148	5.22	0.54
18	152	4.97	167	5.16			0.40
19	122	4.74	144	5.48	125	5.20	0.61
20	146	5.50	171	5.99			0.53
21	120	4.17	150	4.62	114	4.54	0.25
22	135	5.20	160	5.79	140	5.37	0.63
6	141	5.74	199	6.73	165	6.23	0.42
7	138	4.92	214	6.53	134	5.21	0.56
	134	5.21	156	5.82			
8	136	5.10	164	5.70	137	5.45	0.42
23	148	4.14	173	4.51	148	4.17	0.52
Average...	139	4.69	169	5.39	137	4.93	0.54

To escape the difficulty incident to slowing the rate by vagal stimulation it seemed desirable to learn the effect on the consumption of oxygen of driving the heart by electrical stimulation. The heart was driven by rhythmic break shocks in a manner formerly described (6) (7). One electrode was imbedded in the inter-costal muscles, the other was a light

pincette attached to the appendix of the right auricle. That the circuit was operative, was documented by the appearance of induction shocks in electrocardiograms; and was obvious because increasing the rate of the stimuli without changing their strength increased the frequency of the cardiac beat. These experiments show that electrical stimulation of the isolated heart approximately at its spontaneous rate does not change the rate of metabolism (dogs 1, 5, table 1).

In twenty-one experiments in which the cardiac rate was temporarily increased in the manner just described, the consumption of oxygen was measured before, during and after the period of stimulation. The output of the heart and venous pressure varied, but the extent of the change was never great nor was the direction always the same. The temperature usually did not fluctuate more than 0.3°C. In the control periods it averaged 38.1 and in the periods of stimulation, 38.2. Changes due to the effect of temperature on metabolism seem in consequence, negligible.

After the period of stimulation the consumption of oxygen was in certain cases greater and the heart rate slower than in the period preceding stimulation (table 1). This was usually the course of events in those heart-lung preparations which were beginning to fail. Because of this fact the results during the period of stimulation were compared with the results obtained on averaging the figures in the periods preceding and following stimulation.

Results. The consequences of increasing the rate of contraction are fairly clear. The rate of change in consumption of oxygen was proportionately one half as fast as that of frequency of the beat. The relation is more accurately expressed by the linear equation $O_2 = 0.0187 R + 2.23$ where O_2 = cubic centimeters oxygen per gram heart per hour and R = rate per minute. Since it was shown in the preliminary experiments that electrical stimuli of the same frequency as the cardiac rate did not change the rate of metabolism, the increase observed in the rate of utilization of oxygen can be attributed only to changes in frequency and to those other changes such as alteration in diastolic volume known to be dependent on changes in rate.

Comment. Unexpected confirmation of the accuracy of these results was obtained from another source (8). In fifty-four heart lung preparations the natural or uninfluenced rate of beating and the rate of consumption of oxygen were correlated. The coefficient of correlation was found to be $+0.4706 \pm 0.05$. The linear equation representing this relation is $O_2 = 0.0196 R + 1.60$. The curve derived from this equation and those from the standard error of estimate are drawn on the scatter diagram (fig. 1) as well as the curve derived from the results obtained on electrical stimulation (dotted line). The slopes of the two are in close agreement. The slightly higher values for the consumption of oxygen on electrical

stimulation may be attributed to the use of preparations after they began to deteriorate.

An attempt to dissociate the effect of change in rate from such accompaniments as change in diastolic volume, in venous pressure and in cardiac output, was not made, since the sole object in this investigation was to ascertain to what extent change in the consumption of oxygen resulted both directly and indirectly from change in frequency of contraction. Yet such alterations in frequency as were effected did not change ma-

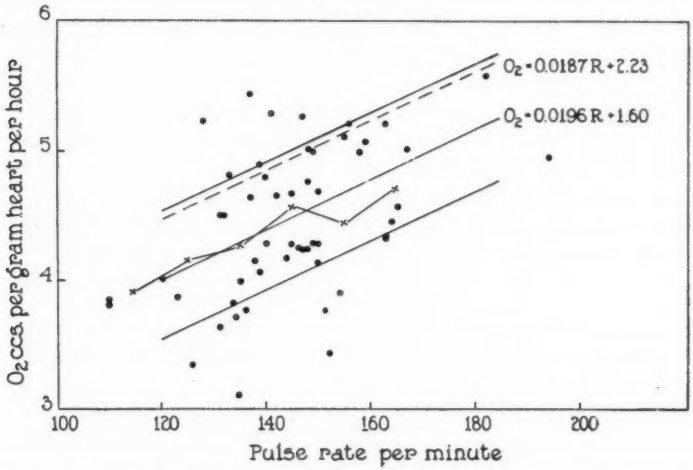


Fig. 1. A scatter diagram is shown of points representing values for rate of consumption of oxygen and frequency of heart beat observed in fifty-three different heart-lung preparations; the continuous curve in the middle is the regression line of consumption of oxygen on frequency of beat. The upper and lower ones mark the magnitude of the standard error of estimate. The crosses signify average consumption of oxygen for observations in which pulse rates fell within a range of 10 beats per minute of each other beginning at 110–120 and ending with 160–170. The dotted curve is the regression of consumption of oxygen on frequency of beat as calculated from altering the rate by rhythmic electrical stimuli.

terially either the cardiac output or the venous pressure. To avoid unforseeable disturbances the volume of the heart was not measured. Sometimes, especially at higher rates (changing from 150 to 180) the cardiac output fell and the venous pressure increased slightly. Often at lower rates (increasing from 110 to 150) the reverse occurred. Occasionally very little change in either volume output or venous pressure took place.

Other considerations lead also to the belief that these results are reasonable. If the relations between frequency and consumption of oxygen are linear, extrapolated curves cross the Y axis at 2.23 and 1.60 cc. of

oxygen. Evans (2) obtained similar results (1.7 and 1.8 cc. in two experiments) although in his experiments at extremes of temperature the relations were not linear. Approaching the question from an entirely different angle Rhode (9) in 1912, having arrested the heart by perfusing it with fluid free from calcium and potassium, found that its rate of metabolism was reduced about one-half.

SUMMARY

Experiments are described which confirm the observation that the rate at which dogs' hearts beat in heart lung preparations influences the consumption of oxygen directly. The results reported were observed in two sorts of experiment:

1. In those in which the hearts were driven by induction shocks.
2. In others in which they were not further influenced than by the nature of the preparation itself.

In both methods the change in the rate of consumption of oxygen progressed roughly one half as fast as change in the rate of contraction. Electrical stimulation itself had no effect on the rate of metabolism.

REFERENCES

(1) KNOWLTON, F. P. AND E. H. STARLING. J. Physiol. **44**: 206, 1912.
(2) MARTIN. H. N. Studies from Biol. Lab., Johns Hopkins Univ. **2**: 119, 1881.
(3) EVANS, C. L. J. Physiol. **45**: 213, 1912.
(4) EVANS, C. L. J. Physiol. **51**: 91, 1917.
(5) STARLING, E. H. AND M. B. VISSCHER. J. Physiol. **62**: 243, 1926–27.
(6) COHN, A. E. AND R. L. LEVY. Proc. Soc. Exp. Biol. and Med. **19**: 174, 1922.
(7) STEWART, H. J. AND J. H. CRAWFORD. J. Clin. Inv. **3**: 449, 1926.
(8) COHN, A. E. AND J. M. STEELE. (To be published.)
(9) RHODE, E. Arch. f. exper. Path. u. Pharmakol. **68**: 401, 1912.

THE EFFECT OF LIGHT AND OF DARKNESS ON THE THYROID GLAND OF THE RAT

H. S. MAYERSON

From the Laboratory of Physiology in the School of Medicine, Tulane University of Louisiana, New Orleans

Received for publication July 3, 1935

Various observers (1–3) have indicated that lack of sunlight may be an important factor in the etiology of goiter, but attempts to demonstrate thyroid hypertrophy or hyperplasia in animals kept in darkness or deprived of ultraviolet radiation, particularly of the antirachitic portion, however, have yielded contradictory evidence (4–9).

In the present study, extending from November, 1932 through February, 1935, one hundred and ninety albino rats were used. Ninety-six were offspring of stock animals bred and kept in the rat room illuminated by diffuse daylight filtered through window glass. The remainder were offspring of rats kept and bred in a dark room, the only radiation coming from a ruby lamp used for a brief period daily while cleaning, feeding or weighing. The animals were grouped at weaning (21–28 days), weighed weekly, and kept under the different experimental conditions designated in the table until they were from 10 to 16 weeks old. There were 3 series: 1, in which a balanced stock ration rich in iodine (Bal-Ra) was used; 2, in which the animals were fed white bread and fresh milk, a diet relatively poor in iodine and known to favor the production of thyroid hyperplasia in rats (10); and 3, in which the Steenbock rachitogenic (11) and goitrogenic (4) diet was used. Distilled water was given *ad libitum*. Representative animals were sacrified at varying intervals, the thyroids being removed by rapid dissection under ether, weighed immediately, and fixed in Zenker's fluid. Paraffin sections were cut and stained with hematoxylin and eosin.

The results are summarized in the table. There are no significant differences in body growth (12), size or microscopic structure of the thyroid gland. With few exceptions, all of the rats fed the stock diet (series 1) showed non-hyperplastic thyroids irrespective of environment. Likewise with few exceptions the animals fed bread and milk (series 2) showed various degrees of hyperplasia, not corrected by any of the experimental procedures; as a matter of fact the most hyperplastic glands were those of group 5 exposed to unfiltered sunlight (8, 13). In series 3, rickets was

TABLE 1

GROUP NO.	NO. OF ANIMALS	BORN IN	KEPT IN	AVER. WT. AT END OF EXPER. (AGE 16 WKS.)	AVER. WT. THYROID PER 100 GM. BODY WT.	HISTOLOGICAL PICTURE			
						Non-hyperplasia	Slight hyperplasia	Moderate hyperplasia	Marked hyperplasia
Series 1. Stock (Bal-Ra) diet									
				grams	*mgm.*				
1	15	Light	Light	231	7.8	13	2	0	0
2	4	Dark	Light	243	7.8	4	0	0	0
3	14	Light	Dark	225	8.8	10	3	1	0
4	13	Dark	Dark	238	8.7	10	3	0	0
5	6	Light	Light & C arc*	215	8.3	4	1	1	0
6	6	Light	Dark & C arc*	217	7.8	5	1	0	0
7	4	Dark	Light & C arc*	215	9.8	4	0	0	0
8	6	Dark	Dark & C arc*	218	9.7	4	2	0	0
9	4	Dark	Light & 4 hrs. sun daily**	236	8.3	3	1	0	0
10	5	Dark	Dark & 4 hrs. sun daily**	218	7.8	3	2	0	0
Series 2. Bread and milk diet (age 12 wks.)									
1	20	Dark	Dark	203	12.5	4	9	5	2
2	8	Dark	Light	212	13.3	0	4	3	1
3	11	Dark	Dark & C arc*	189	12.4	3	4	2	2
4	4	Dark	Dark & Hg arc†	204	11.3	0	4	0	0
5	5	Dark	Dark & 4 hrs. sun daily‡	195	12.3	0	1	0	4
6	5	Dark	Light & sun through window glass♭	196	12.5	0	4	1	0
7	5	Dark	Light & sun§ through G 986 A	209	12.0	0	2	2	1
Series 3. Steenbock rachitic diet (age 10 wks.)									
1	14	Light	Dark	95	21.3	0	4	6	4
2	8	Light	Dark & C arc*	107	14.6	0	5	3	0
3	6	Light	Dark & Hg arc†	105	16.6	0	0	3	3
4	6	Light	Dark & Hg arc□ through G 986 A	95	14.4	0	0	3	3
5	13	Light	Dark + viosterol●	107	20.3	0	6	4	3
6	8	Light	Dark + KI‖	102	11.7	6	2	0	0

* Irradiated thrice weekly, 15 minutes at 1 M from carbon arc (25 A, 45–50 V, "Sunshine" carbons). Total energy value each exposure = 6.51 g. cal. per sq. cm., of which 0.273 g. cal. per sq. cm. = ultraviolet and 0.020 g. cal. per sq. cm. = antirachitic (< 313 mμ) radiation.

** Total 160 hours. Each exposure as estimated from pyrheliometric measure-

ments = 168 g. cal. per sq. cm., of which approximately 16.8 g. cal. per sq. cm. = ultraviolet and 0.192 g. cal. per sq. cm. = antirachitic radiation.

† Irradiated thrice weekly, 15 minutes at 1 M from Uviarc quartz mercury vapor arc (5–6 A, 70–80 V). Total energy value each exposure = 0.591 g. cal. per sq. cm. of which 0.041 g. cal. per sq. cm. = ultraviolet and 0.0059 g. cal. per sq. cm. = antirachitic radiation.

‡ Total 180 hours. Each exposure estimated equivalent to 264 g. cal. per sq. cm., of which about 26.4 g. cal. per sq. cm. = ultraviolet, and 0.50 g. cal. per sq. cm. = antirachitic radiation.

♭ All radiation shorter than 310 mμ absorbed by window glass. Total energy of each exposure same as in ‡.

§ Only radiation, 255–420 mμ, transmitted. Energy-equivalent same as in ‡.

□ See (§). Energy-equivalent same as in group 3.

● Diet supplemented with 0.05 per cent Viosterol 250 D.

‖ Diet supplemented with 0.00128 gram KI per 100 grams of diet.

found only in group 6, fed the KI supplement, and marked hyperplasia and hypertrophy was evident in all except this group. The addition of Viosterol (group 5), while protecting the animals against rickets, failed to influence the development of hyperplasia, which finding supports the results of Thompson (14) and Levine, Remington and von Kolnitz (15), and contradicts those of Nitschke (5) and Bennholdt-Thomsen and Wellman (6).

SUMMARY

Rats kept in the dark, whether born and reared or placed there at weaning, when fed a well-balanced diet, show no differences in rate of growth, body weight, or in the microscopic structure of the thyroid gland when compared with rats kept in diffuse roomlight or exposed to direct sunlight, carbon or quartz mercury vapor arc radiation.

Darkness does not enhance and radiation does not perceptibly modify the hyperplasia produced by feeding goitrogenic diets to rats.

The author is pleased to acknowledge his indebtedness to Dr. C. H. H. Branch, Jr. for technical assistance in the early part of the investigation. The Viosterol used in series 3 was generously supplied by Mead, Johnson & Co. through the courtesy of Dr. C. E. Bills.

REFERENCES

(1) Bernhard, O. Light treatment in surgery. Transl. by R. K. Brown, London, 1926.

(2) Aschoff, L. Proc. Internatl. Congress on Goiter. P. 1, 1927. Quoted by McCarrison, 1932.

(3) Smith, J. H. Arch. Int. Med. **50**: 76, 1932.

(4) Levine, H. and R. E. Remington. J. Chem. Educ. **10**: 649, 1933.

(5) Nitschke, A. Klin. Wchnschr. **12**: 1793, 1933.

(6) BENNHOLDT-THOMSEN, C. AND M. WELLMAN. Klin. Wchnschr. **13**: 800, 1934.
(7) HIGGINS, G. M., C. SHEARD AND R. M. WILDER. Anat. Rec. **58**: 205, 1934.
(8) MCCARRISON, R. Ind. J. Med. Res. **20**: 633, 1932.
(9) KENYON, A. T. Am. J. Path. **9**: 347, 1933; Proc. Soc. Exp. Biol. Med. **32**: 697, 1935.
(10) DRENNAN, A. M., J. MALCOLM AND G. A. COX. Brit. J. Exp. Path. **12**: 430, 1931.
(11) STEENBOCK, H. AND A. BLACK. J. Biol. Chem. **64**: 263, 1925.
(12) BURR, G. O. AND M. SUTERMEISTER. Proc. Soc. Exp. Biol. Med. **29**: 1035, 1932.
(13) KRAUSS, W. E. AND C. F. MONROE. J. Biol. Chem. **89**: 581, 1930.
(14) THOMPSON, J. J. Nutr. **5**: 359, 1932.
(15) LEVINE, H., R. E. REMINGTON AND H. VON KOLNITZ. J. Nutr. **6**: 325, 1933.

INHIBITION FROM THE CEREBRAL CORTEX

D. McK. RIOCH AND A. ROSENBLUETH

From the Department of Anatomy and the Laboratories of Physiology in the Harvard Medical School

Received for publication July 13, 1935

That the cerebral cortex may exert an "inhibitory" effect upon the activity of lower centers has been surmised for some time, but few observations have been made on specific inhibitory effects and their localization. Indirect evidence for "inhibitory" activity of the cortex has been derived largely from ablation experiments, the resulting over-activity in lower centers being interpreted as a "release" phenomenon. Direct evidence of inhibition in animals with the cortex intact has been more difficult to obtain. Simonoff (1866) reported that in young dogs a stronger stimulus was required to produce withdrawal of the leg during electrical stimulation of the frontal portion of the forebrain than before or after the cortical activation. Bubnoff and Heidenhain (1881) published graphic records showing inhibition of a tonic contraction of the M. ext. digit. com. long. in dogs under morphine anesthesia following weak electrical stimulation of the contralateral motor area of the cortex. Stronger stimuli to the identical cortical points caused contraction of the muscle. These authors stated that inhibition was also obtained by properly graded stimuli to other areas of the contralateral cortex and to the "hindbrain." Exner (1882) noted a similar inhibition in dogs and rabbits, but failed to record it. He obtained no inhibition on stimulation of cortical areas distant from motor area or of the cerebellum. Stimulation of the ipsilateral motor area did not inhibit, but facilitated a flexor reflex in the forelimb. Libertini and Fano (1895) reported that stimulation of the frontal lobe in unanesthetized dogs induced a decrease in magnitude, duration and after-effects, and an increase in latency of the withdrawal response of the limbs. The contralateral effects were more marked than the ipsilateral, which were totally absent in the hind limb. Sherrington (1906) noted that frequently on stimulation of the cortex, before contraction of a flexor muscle occurred, there was a widespread relaxation of "tone" in the extensors of the limb. Sherrington's observations were concerned only with the limbs contralateral to the cerebral hemisphere stimulated. Later Graham Brown and Sherrington (1912) found often inhibition more prominent than excitation. The relatively small number of observations on

the inhibitory effects produced by electrical stimulation of the cortex is probably to be explained by the fact that inhibition of motoneurones can be demonstrated only against a background of activity. Graphic records from single muscles are essential in order to demonstrate slight effects, whether inhibitory or excitatory. It is possible that inhibitory effects have been overlooked in some of the studies published because of failure to meet these requirements.

In the present study activity of certain muscles of the hind limbs was evoked either by stimulation of the cortex or reflexly. The effects on this activity of stimulation of the ipsilateral and the contralateral cortices were recorded.

METHOD. Cats, dogs and monkeys (Macaca mulatta) were used. The anesthetics employed were dial (Ciba, 0.65 cc. per kgm. intraperitoneally for cats and dogs, and 0.5 cc. for monkeys), chloralose (0.1 gm. per kgm. intravenously) or urethane (1.0 to 1.5 gm. per kgm. intravenously). Ether anesthesia was administered for the intravenous injections in the cats and monkeys.

The experimental procedure was the following. After anesthetization the frontal cortex, including the motor-sensory area, was exposed on one or both sides. One or two hind-leg muscles were dissected free for recording. The hind legs were otherwise practically immobilized by nerve and tendon section and were fixed by drills through bones. The muscles under observation were attached by hooks through the tendons to levers so that they pulled against stiff rubber bands. Straws on the opposite arm of the levers recorded on a smoked drum. The magnification was from 7 to 8. The maximum shortening of the muscles was less than 1 cm. Since our interest was exclusively in the qualitative aspect of the problem and since such a myograph is inadequate for quantitative studies, no calibrations were made. The muscles used were tibialis anticus, gastrocnemius-soleus, gracilis, semitendinosus, biceps femoris and quadriceps.

Shielded electrodes were applied to the central ends of cut spinal nerves so as to evoke ipsilateral or crossed reflexes in the muscles under investi-

intermittently by hand, the cortex being covered between observations by cotton moist with warm Ringer's solution.

RESULTS. I. *Cats.* Inhibition of the activity of either flexor or extensor muscles—evoked by cortical stimulation or reflexly—was readily obtained by stimulation of the ipsilateral or the contralateral cortex.

Figure 1 illustrates inhibition from the ipsilateral cortex of the contraction of tibialis anticus elicited by stimulation of the contralateral cortex. Figure 2A offers instances showing inhibition of ipsilateral flexor; B, my-

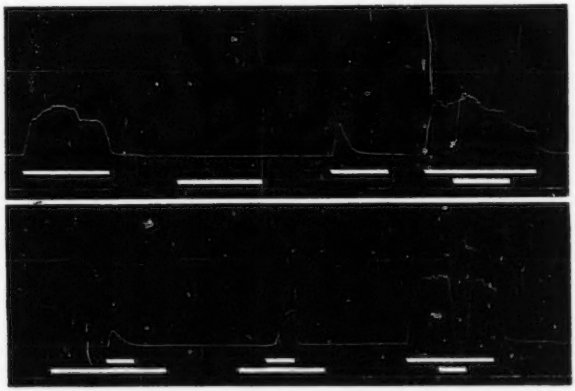


Fig. 1. Cat. Dial. Right cerebellar cortex removed 10 days previously. Upper record: right tibialis anticus. Lower record: left tibialis anticus. Upper signal: stimulation of right (R) cerebral cortex. Lower signal: stimulation of left (L) cerebral cortex. In this and the succeeding figures, the time signal (lowest tracing) records 5-second intervals. The figures in parentheses denote the coil distance of the inductorium in centimeters. For the symbols denoting the cortical areas, see text. The small letters refer to the successive records. a, R area *F* (7); b, L area *F* (7); c, R area *A* (7); d, L gyrus suprasylvius anterior (6.5) and R area *F* (7); e, L area *F* (7) and R area *A* (7); f, L area *F* (7) and R area *F* (7); g, R area *F* (7) and L area *F* (7).

the inhibitory effects produced by electrical stimulation of the cortex is probably to be explained by the fact that inhibition of motoneurones can be demonstrated only against a background of activity. Graphic records from single muscles are essential in order to demonstrate slight effects, whether inhibitory or excitatory. It is possible that inhibitory effects have been overlooked in some of the studies published because of failure to meet these requirements.

In the present study activity of certain muscles of the hind limbs was evoked either by stimulation of the cortex or reflexly. The effects on this activity of stimulation of the ipsilateral and the contralateral cortices were recorded.

METHOD. Cats, dogs and monkeys (Macaca mulatta) were used. The anesthetics employed were dial (Ciba, 0.65 cc. per kgm. intraperitoneally for cats and dogs, and 0.5 cc. for monkeys), chloralose (0.1 gm. per kgm. intravenously) or urethane (1.0 to 1.5 gm. per kgm. intravenously). Ether anesthesia was administered for the intravenous injections in the cats and monkeys.

The experimental procedure was the following. After anesthetization the frontal cortex, including the motor-sensory area, was exposed on one or both sides. One or two hind-leg muscles were dissected free for recording. The hind legs were otherwise practically immobilized by nerve and tendon section and were fixed by drills through bones. The muscles under observation were attached by hooks through the tendons to levers so that they pulled against stiff rubber bands. Straws on the opposite arm of the levers recorded on a smoked drum. The magnification was from 7 to 8. The maximum shortening of the muscles was less than 1 cm. Since our interest was exclusively in the qualitative aspect of the problem and since such a myograph is inadequate for quantitative studies, no calibrations were made. The muscles used were tibialis anticus, gastrocnemius-soleus, gracilis, semitendinosus, biceps femoris and quadriceps.

Shielded electrodes were applied to the central ends of cut spinal nerves so as to evoke ipsilateral or crossed reflexes in the muscles under investigation. Either the saphenous, tibial, peroneal or hamstring nerves were used.

The cortex was stimulated by induction shocks at tetanizing frequency (Harvard induction coil with 2 volts in the primary circuit). The nerves for eliciting the spinal reflexes were stimulated either by single induction shocks, short tetani or condenser discharges timed by a metronome. Control observations were made to eliminate spurious effects from spread of current or from mutual induction when two coils were used simultaneously.

Bipolar stimulation of the cortex by means of silver electrodes (interpolar distance, 2 mm.) was used throughout. The electrodes were applied

intermittently by hand, the cortex being covered between observations by cotton moist with warm Ringer's solution.

RESULTS. I. *Cats.* Inhibition of the activity of either flexor or extensor muscles—evoked by cortical stimulation or reflexly—was readily obtained by stimulation of the ipsilateral or the contralateral cortex.

Figure 1 illustrates inhibition from the ipsilateral cortex of the contraction of tibialis anticus elicited by stimulation of the contralateral cortex. Figure 2A offers instances showing inhibition of ipsilateral flexor; B, my-

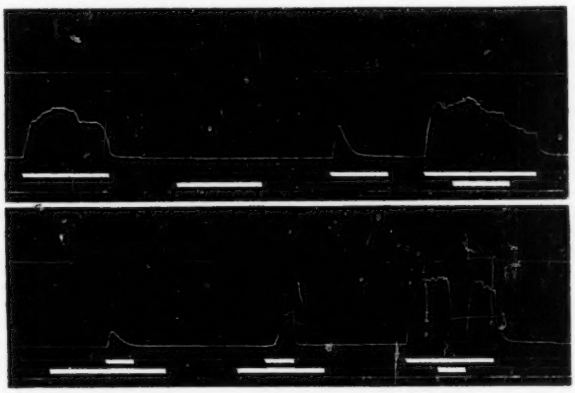


Fig. 1. Cat. Dial. Right cerebellar cortex removed 10 days previously. Upper record: right tibialis anticus. Lower record: left tibialis anticus. Upper signal: stimulation of right (R) cerebral cortex. Lower signal: stimulation of left (L) cerebral cortex. In this and the succeeding figures, the time signal (lowest tracing) records 5-second intervals. The figures in parentheses denote the coil distance of the inductorium in centimeters. For the symbols denoting the cortical areas, see text. The small letters refer to the successive records. a, R area *F* (7); b, L area *F* (7); c, R area *A* (7); d, L gyrus suprasylvius anterior (6.5) and R area *F* (7); e, L area *F* (7) and R area *A* (7); f, L area *F* (7) and R area *F* (7); g, R area *F* (7) and L area *F* (7).

otatic (knee-jerks); and C, D and E, contralateral extensor (crossed extensor) reflexes. Figure 3A shows inhibition of *clonus* of quadriceps.

The regions of the cortex from which inhibition could be evoked were limited to the anterior and posterior sigmoid gyri. Stimulation of the remainder of the frontal and parietal lobes was without effect (figs. 1, 2 and 3) when the intensity of the stimuli was sufficient to produce marked results from these two gyri. In several experiments the threshold for the appearance of inhibition was determined and found to be lowest over the medial third of the anterior sigmoid gyrus (area *A* of Langworthy, 1928). The threshold of Langworthy's areas *B*, *D* and *E* was usually

lower than that of area *F*, but in some instances the reverse was the case. Area *C* gave little or no results. With supraliminal stimuli the inhibition was more marked from the regions of lower threshold (figs. 1, 2 and 3).

The degree of inhibition of all the excitatory responses studied varied not only with the area of the cortex stimulated and with the intensity of the inhibitory stimulus, but also was a function of the duration of this stimulus. Longer periods of stimulation induced more prolonged and more complete relaxation than shorter periods (fig. 3A). The effects recorded depended also on the characteristics of the excitatory stimulus, threshold or nearly threshold excitatory responses being more easily and

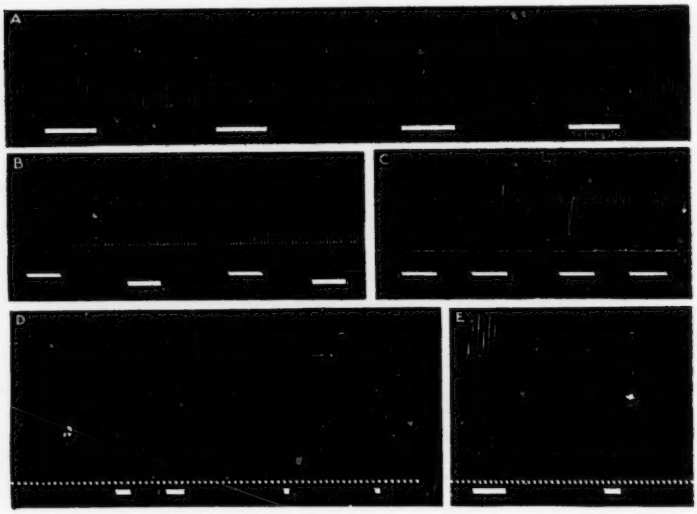


Fig. 2. A. Cat. Dial. Tibialis anticus. R saphenous nerve stimulated centrally by means of condenser discharges timed by a metronome. Cerebral cortex stimulated at signals (7). a, gyrus suprasylvius anterior; b, area *F*; c, area *E*; d, area *A*.

B. Cat. Urethane. Upper record: L gracilis. Lower record: L quadriceps. Knee-jerks elicited regularly with uniform stimulation. Upper signal: L cerebral cortex (7). Lower signal: R cerebral cortex (7). a, L area *A*; b, R area *A*; c, L area *F*; d, R area *F*.

C. Cat. Chloralose. Upper record: R quadriceps. Lower record: R gracilis. Upper signal: R cerebral cortex (7). Lower signal: single induction shocks to L peroneo-popliteal (10). a, gyrus suprasylvius anterior; b, area *F*; c, area *E*; d, area *A*.

D. Same animal and records as in B. Upper signal: repetitive tetanic stimulation of the R peroneo-popliteal (10, timing by a metronome). Lower signal: cerebral cortex (7). a, L area *F*; b, L area *A*; c, R area *F*; d, R area *A*.

E. Same animal, records and signals as in D. a, R area *F*; b, R area *A*.

completely abolished than stronger responses. There was finally considerable variability of the inhibitory action in different animals, at different times in the same animal and for the different responses.

The duration of the after-effects of an inhibitory stimulus was also found to be variable and influenced usually in a similar manner by the factors influencing the degree of inhibition.

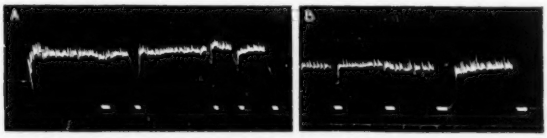


Fig. 3. Cat. Dial. Record of *clonus* of R quadriceps.

A. Signals: cerebral cortex (8). a, R area *F*; b, R area *A*; c, L area *A*; d, L area *F*; e, L area *F* (longer stimulation).

B. Same animal as in A, after removal of the motor and premotor cortices on both sides. Signals: stimulation of the white matter underlying the areas listed (7). a, R area *A*; b, R area *F*; c, L area *A*; d, L area *F*.

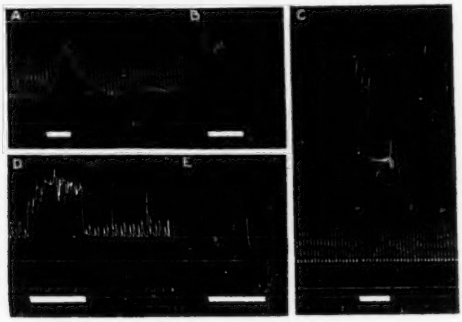


Fig. 4. Cat. Urethane. Upper record: L gracilis. Lower record: L quadriceps.

A. Knee-jerks with constant stimuli timed by a metronome. At signal: stimulation of L area *F* (7). B. L area *F* (7) without knee-jerks. C. Knee-jerks; and at signal: R area *F* (6.5). D. Upper signal: single induction shocks applied to L peroneo-popliteal centrally (10). Lower signal: R area *F* (7). E. R area *F* (7) without stimulation of the peroneo-popliteal.

After removal of the cortex, stimulation of the white matter underlying the areas which yielded inhibition was found to evoke similar effects (fig. 3B). The threshold was higher and the responses quantitatively somewhat less than on stimulation of the intact brain. Stimulation of the neighboring basal ganglia was without effect.

Inhibition of an extensor muscle response was sometimes attended by simultaneous contraction of the antagonistic flexor. This reciprocal in-

nervation effect was especially noticeable on stimulation of the contralateral motor cortex (fig. 4C), which in cats usually evokes flexion. Similarly but more rarely, inhibition of a flexor response was accompanied by contraction of the antagonistic extensor. More frequently, however, particularly when area *A* was stimulated, pure inhibitory effects resulted—i.e., the antagonists did not contract (fig. 2B, C and D). Paradoxical results ensued in some cases. Thus inhibition of a spinal reflex involving a given muscle sometimes occurred simultaneously with contraction of

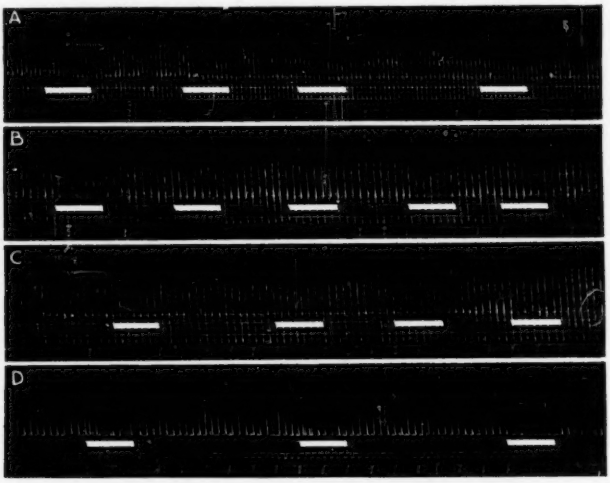


Fig. 5. Cat. Dial. R tibialis anticus. A and B, with cerebellum intact; C and D after complete removal of the cerebellum.

A. Tibialis jerks with constant stimuli. Signals: stimulation of the cerebellum (7). a, R hemisphere; b, L hemisphere; c, vermis, caudal; d, vermis, rostral.

B. Tibialis jerks. Signals: stimulation of R cerebral cortex (7). a, area *A*; b, area *E*; c, area *F*; d, area *A*; e, area *E*.

C. Tibialis jerks. Signals: stimulation of R cerebral cortex (7). a, area *A*; b, area *E*; c, area *F*; d, area *A*.

D. Upper signal: stimulation of R cerebral cortex. Lower signal: single induction shocks applied to R tibial nerve (10). a, area *A*; b, area *E*; c, area *F*.

the same muscle on cortical stimulation (fig. 4A and D). The paradox was particularly striking when a facilitation of the two sets of excitatory nerve impulses (cortical and afferent) reaching the motoneurones was evidenced by the lack of contraction when the cortical stimulus was applied alone (fig. 4B and E).

Simultaneous inhibition of both flexors and extensors occurred in some instances. Such may have been the case when the generalized responses elicited by mechanical stimulation (e.g., tapping the table) in cats under chloralose were completely inhibited by stimulation of area *A* on either

side (see section IV). Finally, an excitatory component could mask an inhibitory effect. Thus in figure 2D brief stimulation of the right area *F* produced inhibition of the crossed extensor response of the left quadriceps, while a similar but more prolonged stimulation in figure 2E led to inhibition succeeded by facilitaion of the reflex and to contraction of the gracilis. The rhythmic character of the response of the latter muscle was due to relaxation during stimulation of the contralateral sciatic.

In the early observations, when only the inhibition from the ipsilateral cortex of the response to the contralateral motor cortex was being studied, the possibility was considered that the corpus callosum might be involved.

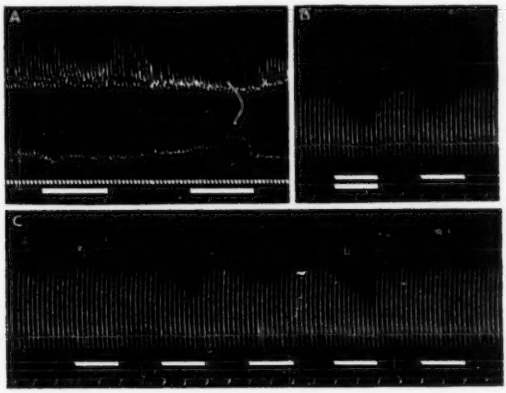


Fig. 6. A. Dog. Chloralose. Upper record: R quadriceps. Lower record: R gracilis. Upper signal: repetitive tetanic stimulation of L peroneo-popliteal. Lower signal: stimulation of R cerebral cortex (6). a, area *A*; b, area *F*.

B. Dog. Urethane. Upper record: L gracilis. Lower record: L quadriceps. Upper signal: stimulation of L area *A* (7). Lower signal: stimulation of *R* area *A* (7). Knee-jerks evoked with constant stimuli.

C. Same animal and records as in B. Knee-jerks. Signals: L cortex (7). a, area *F*; b, area *A*; c, area *G*; d, area *E*; e, meninges.

Transection of the corpus callosum, however, did not abolish this inhibition. Deafferentation of the hind limbs likewise failed to abolish the inhibition.

It was deemed possible that the cerebellum might be involved in the path of the inhibitory cortical influence. Stimulation of the cerebellum was found to inhibit all the responses studied. No clear localization was apparent other than that the inhibition was somewhat more pronounced from the vermis than from the lateral lobes and that the rostral part of the vermis was more effective than the caudal (fig. 5A). Three cats were studied, in the first of which the cerebellar vermis had been removed several months previously; in the second, the cortex of the right cerebellar

hemisphere had been ablated without section of the peduncles (fig. 1); and, in the third, the right half of the cerebellum had been removed one week previously. In the three animals the inhibitory effects from the homolateral cerebral cortex were still clearly present. Complete acute removal of the cerebellum was finally found to leave the inhibition from the cerebral cortex unaltered (fig. 5B, C and D).

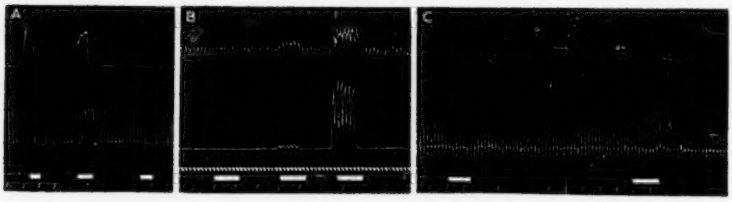


Fig. 7. A. Dog. Urethane. Upper record: L gracilis. Lower record: L quadriceps. Knee-jerks. Signals: R cerebral cortex (8). a, area *A*; b, area *F*; c, area *E*.

B. Same animal and records as in A. Upper signal: repetitive tetanic stimulation of L peroneo-popliteal. Lower signal: R cerebral cortex (7.5). a, area *A*; b, area *E*; c, area *F*.

C. Dog. Chloralose. Records: as in A and B. Knee-jerks. Signals: L cerebral cortex (7). a, area *A*; b, area *F*.

Fig. 8. Monkey. Urethane. Upper record: L semitendinosus. Lower record: L quadriceps. Upper signal: stimulation of cerebral cortices (7). Lower signal: repetitive tetanic stimulation of L saphenous (8). a, control; b, L area 6; c, R area 6; d, control; e, L area 4 (leg); f, R area 4 (leg); g, R area 4 (leg) without saphenous stimulation.

In order to determine the spinal path of the inhibitory tracts, observations were made before and after sections of the cord in the midthoracic region that involved two or three quadrants. In evaluating these results only positive data were considered significant. The extent of the sections was determined macroscopically after fixation of the cords in formalin.

After a right semisection of the spinal cord, stimulation of area *A* in the left cerebral cortex inhibited both hind limbs. The inhibition from stimulation of the same area in the right cortex was also bilateral but

markedly less on the right leg. After section of both dorsal quadrants stimulation of area *A* in either cortex produced inhibition in both legs; no motor effects occurred after this section. Sections of three quadrants confirmed the previous observations. Thus, with only one ventral quadrant intact, inhibition from the ipsilateral cortex was still recorded in both legs.

II. *Dogs.* Similar but more complex and less consistent inhibitory effects of cortical stimulation were found in dogs. Illustrative examples are given in figures 6 and 7. For purposes of localization the sigmoid gyri of the dog will be divided into areas *A*, *B*, *C*, *D*, *E* and *F*, analogous to those described by Langworthy in the cat.

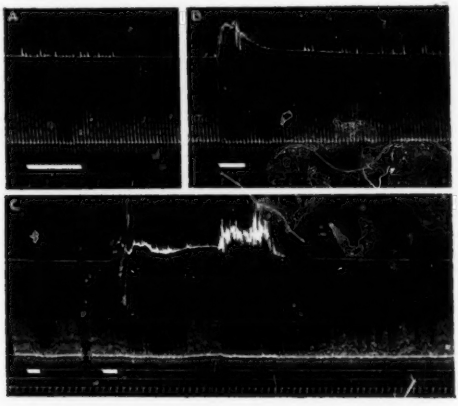


Fig. 9. Monkey. Chloralose. Upper record: L semimembranosus. Lower record: L quadriceps. Knee-jerks. Signals: stimulation of R cerebral cortex (6). A. Area 4 (leg). B. Same, later. C. a, area 6; b, area 4 (leg, a different spot).

In figure 6A contralateral flexor and extensor reflexes are recorded. Stimulation of the ipsilateral area *F* inhibits the extensor and slightly facilitates the flexor response. Stimulation of the ipsilateral area *A* inhibits more markedly the extensor and has a paradoxical dual effect on the flexor activity. In figure 6B simultaneous stimulation of the two areas *A* is more effective in reducing the knee-jerks than stimulation of the contralateral alone. In 6C are shown ipsilateral inhibitory effects.

Figure 7A and B illustrates contralateral effects on reflexes involving an extensor and a flexor muscle respectively. Figure 7C shows ipsilateral inhibition of the knee-jerk from area *A* and facilitation from area *F*.

III. *Monkeys.* The inhibitory effects of cortical stimulation obtained in monkeys were also similar to those in cats, but still more complex than the results in dogs. Figures 8, 9 and 10 illustrate these effects.

Figure 8 demonstrates inhibition of the ipsilateral flexor reflex from the ipsilateral areas *6* and *4* of Brodmann (records b and c); a more complete inhibition (c) from the contralateral area *6*, in sharp contrast with facilitation (b) from the contralateral area *4*.

Figure 9 shows the influence of the contralateral cortex on the knee-jerks. In A the leg area inhibits the knee-jerks and facilitates flexor contractions synchronous with the knee-jerks. In B the excitatory action on the flexor is emphasized, and a typical reciprocal influence ensues. In C, area *6* induced a purely inhibitory effect in the recording leg (a)—there were epileptiform movements in the fore-limbs and head—, while

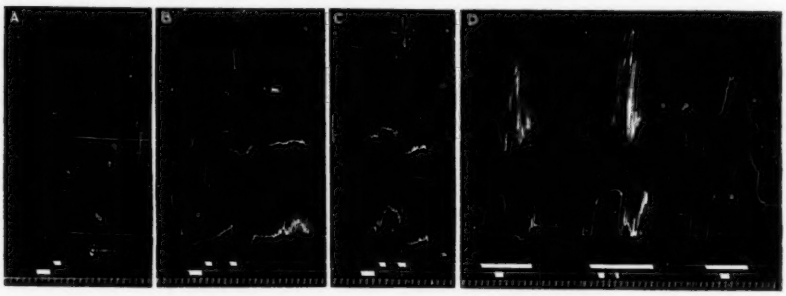


Fig. 10. Monkey. Chloralose. Upper record: L semimembranosus. Lower record: L quadriceps. Signals: stimulation of the cerebral cortices (7) and of the L peroneo-popliteal centrally (9).

A. Control. L area *6* succeeded by R area *1*. B. L area *6*, then R area *1*, then R area *4* (leg). C. L area *6*, then two neighboring spots (*i* and *ii*) in the R area *6*. D. Upper signals: L peroneo-popliteal. Lower signals: cerebral cortices as listed. a, R area *6* (spot *ii*); b, R area *6* (spot *i*), then R area *4* (leg); c, L area *6*.

Drum stopped between the successive observations. Complete relaxation of the muscles after stimulation of the peroneo-popliteal required several minutes.

the leg area (b) evoked epileptiform contractions of the flexor muscle, during which the knee-jerks were inhibited.

Figure 10 illustrates the influence of the cortex on movements of cortical origin (A, B and C) and on ipsilateral flexor and extensor reflexes (D). In A a prolonged contraction of both flexor and extensor muscles was evoked from the ipsilateral area *6*. Stimulation of a contralateral sensory region was without influence on this response. In B stimulation of the contralateral leg area first inhibited the responses to the ipsilateral area *6*, and was followed by an epileptiform after-discharge. In C the responses to the ipsilateral area *6* were facilitated from a point (*i*) and inhibited from a neighboring point (*ii*, 2 mm. distant) in the contralateral homologous area. In D an ipsilateral flexor and extensor response

was inhibited by the contralateral point *ii* (a) and facilitated by point *i* (b). Reciprocal effects were obtained from the contralateral leg area (b). Finally, both the flexor and the extensor responses were enhanced on stimulation of the ipsilateral area *6* (c).

IV. *The anesthetics.* Striking differences were shown by the various animals under the anesthetics employed.

Motor effects from the cortex were easily obtained in all three species when under dial anesthesia (Ciba, containing urethane). In cats (24 animals) the responses from the distal segments of the hind limbs were more readily evoked than those from the proximal segments. In dogs (3 animals) the situation was reversed. In monkeys (2 animals) it was similar to that in the cats but more marked. In cats under dial the face area is limited to area *C*—less extensive than under ether or local anesthesia (unpublished observations). The area from which arm movements may be elicited is more extensive, including area *D*. In the three species flexor responses (tonic) were the rule. No ipsilateral movements were detected in the cats; they did occur in the dogs and monkeys (usually extension).

Motor effects from the cortex under urethane anesthesia were obtained in the three species (2 cats, 1 dog, 2 monkeys). Contraction of the contralateral flexor muscles occurred in all the animals and simultaneously of the extensors in the cats and the dog. The responses differed from those obtained under dial and chloralose in that they showed a clonic character of contraction alternating with partial relaxation. The frequency of the clonic rhythm was about 2 per second in the cat and about 5 per second in the monkey. Stimulation of the inhibitory areas in the cortex not only induced relaxation, but also slowed the rate of the clonus.

Under chloralose only 2 out of 6 cats showed motor responses (flexion or extension) on stimulation of the cortex. In the dogs (3 animals) movements could be readily elicited, usually succeeded by irregular, localized, epileptiform after-effects both tonic and clonic, lasting less than one minute. Finally, in the monkeys (2 animals) the cortex was markedly hyperexcitable from the standpoint of motor responses. In one monkey flexion of the fingers could be easily elicited by a single induction shock applied to the contralateral hand area. Further, faradic stimulation, even of just threshold intensity, resulted in typical epileptiform seizures (fig. 9C). With stronger stimuli these seizures followed the Jacksonian march, became generalized, and lasted frequently as long as 15 minutes. The usual sequence of tonic, then clonic contractions occurred. The flexor muscles were more active than the extensors and were sometimes exclusively involved. Generalized seizures could be induced by stimulation of area *6* as well as area *4*.

As regards the inhibitory effects from the cortex, two facts may be

mentioned. Although motor responses from cortical stimulation in cats under chloralose were seldom obtained (see above), inhibition was readily and consistently elicited (fig. 2C). In the monkey under dial, where good motor responses were evoked, no inhibition could be demonstrated.

Finally, with reference to the spinal reflexes certain features may be noted. Under dial anesthesia, while ipsilateral reflexes (flexor, myotatic and sometimes extensor) were active (figs. 2A and 3), the contralateral reflexes were absent. Under urethane ipsilateral and contralateral flexor and extensor reflexes were obtained. These showed a most striking characteristic in that the after-discharge was very prolonged as compared to the corresponding reflexes in the spinal animal (figs. 2D and E, 4D and 8). The crossed extensor reflex, elicited by a short tetanus, frequently lasted as long as 15 to 20 seconds. Under chloralose, as under urethane, ipsilateral and contralateral flexor and extensor reflexes were found (figs. 2C, 6A and 10D). The flexor reflex was not as consistent as the crossed extensor. The myotatic reflexes were very active. The generalized response to mechanical stimulation, well known in cats and dogs, did not appear in the two monkeys observed.

Discussion. On stimulation of certain regions of the cerebral cortex it is possible to obtain simultaneous contraction of antagonistic muscles (figs. 7B and 10A). It is also possible to induce relaxation of the antagonists associated with contraction of the agonists (fig. 4C). It is finally possible to inhibit simultaneously both flexor and extensor muscles or to inhibit either without producing contraction in the antagonist (figs. 2, 6, 10B and C).

The interpretation of these experimental results is difficult, because of the following complicating factors. Stimulation of a given area probably involves simultaneous activation of elements with antagonistic functions. Further, the effects of the corticofugal impulses will depend upon the action of impulses from other regions coincidentally converging on the final common path. Thus the experimental conditions will markedly influence the results, e.g., the different effects obtained under different anesthetics (section IV).

Although any generalization can therefore only be tentative, it may be suggested that there is a correlation between these experimental results and the behavior of the intact animal in which fixation of a limb (simultaneous contraction of antagonists), movement (reciprocal innervation), or complete relaxation are seen. Hence, it is probable that excitation and inhibition of cortical origin may occur independently.

In considering localization of the inhibitory functions of the cortex, the following schematic systematization is suggested by the data presented. In order to simplify the nomenclature the term "motor area" will be used to denote the Betz cell area, and "premotor area" to denote

the rostrally adjacent agranular cortex (cf. Fulton and Kennard, 1934). In the three species studied the contralateral motor area usually produces reciprocal inhibition. In the cat both the ipsilateral and the contralateral premotor areas inhibit both flexors and extensors; the ipsilateral motor area has similar but less marked effects. In the dog and monkey the same areas exert a similar inhibitory influence, but it is more difficult to demonstrate than in the cat (pp. 671, 672). From this standpoint the dog occupies an intermediate position between the cat and the monkey. It is noteworthy that both inhibitory and motor representations are bilateral in the three species.

The spinal path of the inhibitory tracts in the cat is probably in the ventral quadrants, possibly in the direct pyramidal tract. Since inhibition from both cortices on both hind limbs was recorded after semisection of the cord (p. 670) there must be cross connections below the section. It is possible that these cross connections may be effected through internuncial neurones, since in some of the experiments evidence was obtained that inhibition is sometimes not exerted directly on the motoneurones (see below).

The paradoxical effects encountered, when simultaneous inhibition and facilitation occurred (p. 668, figs. 4 and 7), require further study for a detailed interpretation. In such instances the inhibition cannot have been exerted on the motoneurones directly, but must have occurred higher upstream in the reflex arc, at some internuncial neurone; for if the motoneurones had been directly inhibited the facilitation would not have been demonstrable. The data do not permit any conclusion as to whether the internuncial neurones were invariably the site of inhibition. In other instances the motoneurones might well have been directly inhibited.

It is interesting to note that in the monkeys under chloralose during the epileptiform after-discharges following cortical stimulation all spinal reflexes were inhibited. This was invariably observed, whether the recording muscle or its antagonist was involved in the seizure or not—i.e., when the seizure was limited to other regions of the body (fig. 9).

The marked differences in the results obtained with the three anesthetics employed suggest the following considerations. The responses to cortical stimulation in a given species varied in type (tonic, clonic, epileptiform; see p. 673). The localization in the cortex varied also (p. 673). Excitatory and inhibitory effects were affected independently (cf. particularly the cats under chloralose, p. 673). It is therefore apparent that a study of the localization of function in the cerebral cortex with any one anesthetic may lead to an incomplete and disproportionate view. The spinal reflexes recorded were also unequally influenced by the anesthesia (p. 674). Obviously different anesthetics affect different structures in the central nervous system to different degrees.

SUMMARY

Using as an excitatory background the motor responses of a hind limb, elicited reflexly or by contralateral cortical stimulation, inhibition from the cortex was studied in cats, dogs and monkeys under dial, chloralose or urethane anesthesia. Bilateral inhibitory effects were obtained from either hemisphere in the three species (figs. 1 to 10). This inhibition was more readily obtained in the cat from the *area frontalis agranularis* than from the Betz cell area (p. 675).

Several lesions of the central nervous system were performed to determine the probable path of the inhibitory tracts in the cat. Evidence was obtained that the corpus callosum and the cerebellum are not involved and that the spinal path is mainly in the anterior columns. Crossing over was demonstrable below the midthoracic region (pp. 670, 675).

The independence of the excitatory and the inhibitory effects which may be evoked by stimulation of the cerebral cortex is discussed (p. 674).

The influence of the several anesthetics employed on motor and inhibitory effects from the cortex and on spinal reflexes in the three species is described (p. 673) and discussed (p. 675).

REFERENCES

Brown, T. Graham and C. S. Sherrington. Proc. Roy. Soc. Series B. **85**: 250, 1912.

Bubnoff, N. and R. Heidenhain. Pflüger's Arch. **26**: 137, 1881.

Exner, S. Pflüger's Arch. **28**: 487, 1882.

Fulton, J. F. and M. A. Kennard. Localization of function in the cerebral cortex, p. 158. Baltimore, 1934.

Langworthy, O. R. Bull. Johns Hopkins Hosp. **42**: 20, 1928.

Libertini, G. and G. Fano. Arch. ital. de biol. **24**: 438, 1895.

Sherrington, C. S. The integrative action of the nervous system. New Haven. 1906.

Simonoff, L. N. Arch. f. Anat. 545, 1866.

FACTORS CONCERNED IN THE ARREST OF CONTRACTION IN AN ISCHEMIC MYOCARDIAL AREA[1]

ROBERT TENNANT[2]

From the Department of Physiology, Western Reserve University Medical School, Cleveland, O.

Received for publication July 15, 1935

In a previous communication (Tennant and Wiggers, 1935) it was shown that after ligation of the ramus descendens anterior the myogram recorded from the ischemic region exhibits an evoluting series of changes which lead in an interval of approximately one minute to abrogation of contraction so that each systolic rise of intraventricular pressure causes an actual expansion of the affected zone.

The mechanisms involved in such rapid contractile failure are of great interest particularly since their understanding might throw further light on the biochemical factors responsible for cardiac contraction. In some respects, the myographic method with its ability to record the state of contraction from beat to beat in the intact heart offers a better method for evaluating biochemical changes in the myocardium than do the usual chemical studies on excised tissues. Thus the effect of injecting directly into the coronary circulation substances such as sodium cyanide, monoiodoacetic acid and potassium chloride or of perfusing the coronary artery with blood containing an excess of the various known products of muscle metabolism, e.g., sodium lactate, or pH change, can readily be studied.

PROCEDURE. Dogs were anesthetized with morphine and sodium barbital and the heart was exposed in the customary manner. The myographic apparatus and technique employed in the present experiments were the same as those previously described except that in addition a small side branch of the ramus descendens anterior was cannulated for introduction of the various substances into the coronary circulation. In some instances electrocardiograms (lead 2) were taken. Simultaneous aortic pressure pulses were always recorded with calibrated optical manometers. Optical records were taken before and at frequent intervals after the injection of the various substances studied, and in some experiments continuous records were taken. In each experiment the effect of several different substances was tested, although in each instance the succeeding injection

[1] The expenses of this investigation were defrayed by a grant from the Josiah Macy, Jr., Foundation.

[2] Fellow of the National Research Council.

was not made until the myogram revealed complete recovery of contraction in the affected zone.

EXPERIMENTAL RESULTS. *Potassium chloride.* In four experiments 2 cc. of 5 per cent KCl were injected into a coronary side branch. In every instance the myogram showed complete inversion (fig. 1). The abrogation of contraction occurred instantaneously upon the injection of the drug and without the evoluting changes seen in the coronary ligation experiments. With the deletion of these fractionate contractions the duration of ventricular systole as a whole was shortened as in the ligation experiments. There was usually marked slowing of the heart rate as part of the chemical entered the general circulation. The electrocardiograms showed alterations in conduction, although their form was variable. Ventricular fibrillation terminated three of these experiments before recovery of contraction supervened. One of these was revived by use of electric countershocks, with quick restoration of normal contraction. In the fourth experiment recovery occurred gradually about 5 minutes after the injection of the salt. With return of contraction the electrocardiogram was restored to the normal form.

Sodium cyanide. The effect of sodium cyanide injected directly into the coronary circulation in doses varying from 1 to 3 mgm. (solution in saline 1 cc. = 1 mgm.) was studied in 10 experiments. The myograms from the affected region showed an evoluting series of changes in contour leading after an interval of approximately 1 minute to inversion of the contraction curve (fig. 2). In several instances a marked slowing of the rate of the heart was also observed and sometimes irregularity with frequent extrasystoles. The electrocardiogram usually showed no alteration except in one experiment in which it exhibited a high takeoff of the S-T segment from the R-wave. After an interval of from 3 to 5 minutes complete return of contractile power occurred, except in two instances in which ventricular fibrillation terminated the experiment.

It is apparent from these results that sodium cyanide which prevents oxidation in the tissues by inhibiting the oxidative enzymes, produces effects on myocardial contraction in every way comparable to those of anoxemia produced by ligation of the coronary artery.

Monoiodoacetic acid. The effect of monoiodoacetic acid on myocardial contraction was studied in 19 experiments. The drug was injected in doses from 1.5 to 10 mgm. (solution in saline 1 cc. = 2 mgm.). In 13 experiments the unneutralized acid was injected *via* the side branch and in 6 experiments sodium monoiodoacetate, made by neutralizing the acid with Na_2CO_3. The myograms showed the presence of contraction in all except two of these experiments. The only alterations observed were a slight diminution in the amplitude of contractions in a few instances. The electrocardiograms recorded showed no changes. In the first of the two

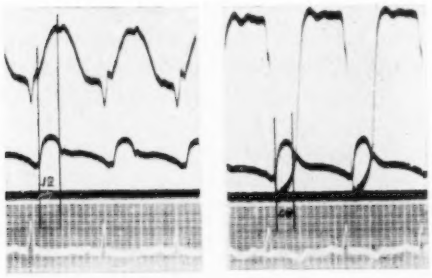


FIG. 1

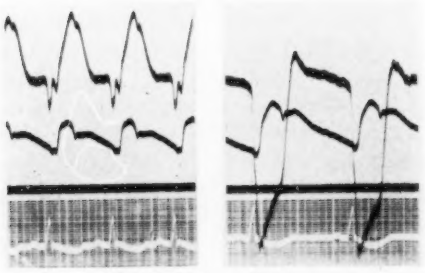


FIG. 2

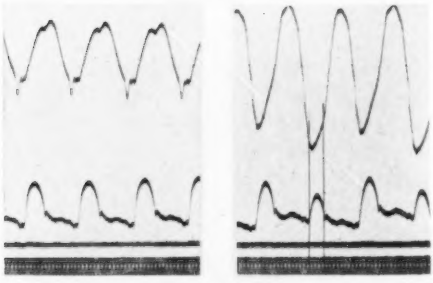


FIG. 3

Fig. 1. Myograms (upper curve) before and after injection of potassium chloride recorded simultaneously with aortic pressure (middle) and electrocardiograms lead 2. Discussion in text.

Fig. 2. Registration same as figure 1, showing in second curve inversion of myogram after use of sodium cyanide.

Fig. 3. Myograms (upper curve) before and after perfusion of coronary artery with blood-Locke-lactate solution showing inversion of myogram. Lower curve aortic pressure. Time 0.02 second.

exceptions with a dose of 4 mgm. of iodo-acetic acid the myograms showed the typical effects of coronary ligation with inversion persisting for 30 minutes after the administration of the drug. This experiment was the first of the series and adequate precautions had not been taken to prevent clotting in the side branch cannula. It was suspected that with the injection of the drug a clot from the cannula was forced into the vessel producing coronary obstruction. In the second exception inversion also occurred, but partial recovery was already present when ventricular fibrillation terminated the experiment 4 minutes after the injection. The dose in this case was 8 mgm. of the unneutralized acid. It seems probable here that the failure of contraction was associated with a lowering of the pH of the tissues following introduction of a relatively large dose of this fairly strong acid. Further support for this explanation comes from the six experiments with the neutralized acid where doses of 8 and 10 mgm., sometimes repeated 2 or 3 times in the same animal, were uniformly without significant change in the contraction curve.

The lack of any significant change in contraction following use of monoiodoacetate is in keeping with the results of Clark, Eggleton and Eggleton (1932) who found that frogs' hearts perfused with Ringer's solution containing concentrations of iodoacetate up to 1:5000 had no effect on the contraction for periods as long as 2 hours in the presence of oxygen.

Sodium lactate. One hundred cubic centimeters of blood (heparinized) were drawn from the animal and this was diluted with an equal volume of Locke's solution. The diluted blood was then divided into two equal portions of 100 cc. each. To one of these sodium lactate was added to make a concentration of 400 mgm. of sodium lactate per 100 cc. of solution. With the ramus descendens anterior clamped, the peripheral portions of the coronary artery were perfused *via* the side branch first with blood-Locke's solution and then with blood-Locke-lactate solution. Each perfusion lasted approximately 50 seconds and just before the finish of each, the coronary clamp was released so that the normal circulation was reëstablished without interruption of blood supply at any time. Continuous records were taken throughout the period of perfusion.

In two experiments perfusion with *blood-Locke's solution* was entirely without effect on the contraction curve, while in each instance perfusion with *blood-Locke-lactate solution* caused failure of contraction with inversion of the myogram, as is well illustrated in figure 3 where in addition pulsus alternans had developed. In one the failure was present 9 seconds after the onset of the perfusion, while in the second 20 seconds elapsed before inversion developed. With restoration of normal blood supply at the end of each of these perfusions a prompt complete recovery of contraction was found. These results strongly suggest that an excess of sodium-

lactate is one of the predisposing factors in producing failure of myocardial contraction.

Discussion. The clear-cut myographic demonstration of the abrogation of myocardial contraction in the affected zone following the injection of KCl offers additional evidence of the reliability of the method as an index of the state of muscle shortening in limited regions of the ventricle.

The interesting finding of a shortening of systole concomitant with the deletion of these fractionate contractions further substantiates the correctness of the assumption that the shortening of systole in experimental coronary occlusion is the result of deletion of the muscle fractions in the ischemic zone, as first postulated by Orias (1934) and later confirmed by the myographic method (Tennant and Wiggers, 1935).

The immediate reversal of the myogram following injection of KCl is in accord with our knowledge as to its great diffusibility. While no special study of the mechanisms was made, the electrocardiographic evidence indicated that both failure of conduction and of contraction may be concerned, to varying degrees.

The present experiments show clearly that with the coronary circulation intact contractions in the affected region of the ventricular myocardium are promptly abolished in the presence of an excess of sodium cyanide or sodium lactate, whereas prevention of lactic acid formation by sodium iodoacetate is without effect on myocardial contractions.

While any pretence to a final interpretation of these results is out of the question, particularly in view of the ever changing conceptions of the chemical mechanisms responsible for the energizing of muscular contractions, still it seems worthwhile to attempt an alignment with the known facts concerning cardiac contraction.

Evidence from many sources supports the belief that phosphocreatine and possibly also adenylpyrophosphate breakdown furnish the initial energy for contraction of cardiac as well as skeletal muscle under normal conditions. The absence of any significant change in contraction with sodium iodoacetate injections supports this viewpoint, for certainly the breakdown of glycogen to lactic acid as a source of energy is inhibited under these conditions. It has been shown by Clark, Eggleton and Eggleton (1932), however, that the content of phosphocreatine in the frogs' ventricle is sufficient to supply energy for only twenty beats in the absence of oxygen and hence the maintenance of anaerobic activity for any longer period is dependent on the breakdown of glycogen to lactic acid. That this also occurs in mammalian hearts is indicated by the fact that following experimental ligation of the coronary artery in dogs lactic acid accumulates in the muscle at the expense of glycogen breakdown (Himwich, Goldfarb and Nahum, 1934; Grayzel, Tennant, Sutherland and Stringer, 1934).

The mechanism of the rapid failure of contraction after short periods of anaerobiosis, whether as a result of coronary ligation or NaCN injection, is still an unsettled question. The two views expressed are 1, that the mere accumulation of lactic acid in the muscle inhibits muscular contraction, and 2, that the anaerobic lactic acid formation rapidly utilizes the available base and produces a reduction of the tissue pH so that further lactic acid formation is prevented and muscular contraction ceases.

The striking abrogation of contraction when the coronary artery was perfused with blood containing an excess of lactate with no significant change in pH is strong evidence that excess lactate has a direct effect in inhibiting contraction, although it does *not* exclude change of pH as another possible mechanism. The single instance in the monoiodoacetic acid experiments where a large dose of the unneutralized acid caused myocardial failure is strongly suggestive that pH change is also an important factor.

SUMMARY

1. Simultaneous registration of optical myograms and aortic pressure pulses indicate that abolition of myocardial contraction similar to that following coronary occlusion is produced in the presence of oxygen by potassium chloride and sodium cyanide, but not by sodium iodoacetate.

2. Perfusion of a ventricular zone in the normally beating heart with sodium lactate in buffered blood-Locke's solution similarly arrests contraction. This fact, together with other experimental evidence is strongly suggestive that excess lactate in itself is a factor in preventing contraction under anoxic conditions, although it does not exclude change in pH as another possible mechanism.

REFERENCES

TENNANT, R. AND C. J. WIGGERS. This Journal **112**: 351, 1935.
CLARK, A. J., M. G. EGGLETON AND P. EGGLETON. J. Physiol. **75**: 332, 1932.
ORIAS, O. This Journal **100**: 629, 1932.
HIMWICH, H. E., W. GOLDFARB AND L. H. NAHUM. This Journal **109**: 403, 1934.
GRAYZEL, D. M., R. TENNANT, S. W. STRINGER AND F. A. SUTHERLAND. Proc. Soc. Exper. Biol. and Med. **31**: 837, 1934.

THE INTERPRETATION OF MONOPHASIC ACTION POTENTIALS FROM THE MAMMALIAN VENTRICLE INDICATED BY CHANGES FOLLOWING CORONARY OCCLUSION

HAROLD C. WIGGERS AND CARL J. WIGGERS

From the Department of Physiology, Western Reserve University, Medical School, Cleveland, O.

Received for publication July 15, 1935

The discovery by Orias (1928) that ligation of the ramus descendens anterior promptly and consistently causes an abbreviation of ventricular systole and a reduction of systolic pressure as well, could only be rationalized by assuming an almost immediate deletion of contracting fractions in the ischemic area. It was our expectation, through experiments initiated in 1933, to obtain tangible support for such an assumption, by recording monophasic action currents from the region involved. Regardless of whether one is inclined to favor the long accepted presumption that monophasic currents signal the state of negativity beneath the so-called exploring electrode exclusively—or—whether one champions the new interpretation set forth by Wilson, Macleod and Barker (1) that such monophasic curves are essentially dominated by electrical potential variations at the margin of the dead area—one must admit that they unquestionably represent potential differences between two electrodes so arranged as to lead from an injured and uninjured area.

Consequently, if the potential developed under an exploring electrode on the anterior surface of the left ventricle is significantly altered as a result of a deprivation of the blood supply to this area, while the potential of an original dead area at the right ventricular base remains unaltered, significant changes should occur in the monophasic leads derived. It so developed that the changes in the curves obtained have a definite bearing on the general interpretation of monophasic curves from the ventricular surface.

METHODS. Dogs were anesthetized with morphine and sodium barbital and their chests opened to permit a convenient exposure of the heart. An area of injury was produced on the right basal surface of the right ventricle, quite distant from the interventricular septum, by the method of Schütz (2) i.e., by sucking a bit of right ventricular muscle into a glass tube, about 1 cm. in diameter, and ligating tightly the tuberosity so produced. Probably this tissue will not remain viable for many minutes,

but the spot is certainly dead within thirty minutes after ligation. A wick from a silver chloride electrode was stitched to this dead area. Leads from an uninjured area were made by various types of electrodes—a new form of $AgCl_2$ electrode proving most satisfactory. The latter consisted of a T tube drawn down at one end to a 1 to 2 mm. bore which was held against the heart by a gentle suction applied through the lateral arm. The tube was filled with physiological saline and the upper limb stoppered by a rubber cork, perforated by a silver rod coated with $AgCl_2$. This T tube electrode was maintained in crucial apposition by means of an adjustable clamp. This new electrode had the great advantage that the degree of moisture or dryness of the surface about the electrode had a negligible effect.

Records from these surface points, together with a conventional lead II electrocardiogram, were first registered by two large Hindle galvanometers. In most of the experiments however the G. E. thermionic amplifying electrocardiograph was employed, the galvanometer being removed and mounted upon a solid table, so that monophasic currents could be photographed simultaneously with an E.C.G. for standard reference. The chief advantage gained by use of this instrument lies in the elimination of the difficulties ordinarily experienced as a result of changing resistance. A further advantage lies in the facility of recording curves of large amplitude which allow extraneous deflections and alterations in gradient to be easily discerned.

The ramus descendens anterior was isolated and prepared for the easy application of a small clamp.

The characteristics of normal monophasic curves and their points of reference. Although perfectly smooth monophasic curves can occasionally be obtained from the mammalian heart, these are exceptions rather than the rule. An illustration of an average monophasic curve (obtained by our method) is reproduced in segment I of figure 1. That such curves, and similar ones presented by other investigators, do not strictly represent potential differences limited to minute muscular fractions immediately under the electrodes is evidenced by the occurrence of variations during auricular excitation. Whether the sharp oscillations on the ascending limb of the curve—one of these customarily occurring near the foot and another approximating the peak of the steep rise—are also extrinsic effects may be questioned. However, the fundamental wave upon which the latter are superimposed, unquestionably does represent the potential differences occurring under the electrodes.

In descriptions of monophasic curves they have generally been sectioned vaguely into three constituents, namely, a rise, a plateau and a fall of potential. Accurate examination of any curve, particularly those of large amplitude as recorded by us, reveal most convincingly that the

slopes undergo constant transformation. In segment I, for instance, the curve, subsequent to a minute initial deflection, rises sharply at *A*, culminating in a peak, *B*, within 0.03 second. Following an incisura, the summit *C* is reached, within a total interval of 0.05 second. After this point, the potential difference progressively decreases, moderately (*C-D*), then more rapidly (*D-E*), and still more acutely (*E-F*). Between *F-G*, not only does its approach to isopotential become most rapid, but also, the descending gradient remains unaltered for the longest period. Regardless of the form and amplitude of monophasic curves, the demarcation *F* on the descending limb apparently indicates the precise moment at which the electrical potential differences between the two electrodes decisively seek their state of isopotentiality. We therefore regard this demarcation point, *F*, of as great importance as the moment of rise at *A*.

In the evaluation of potential differences between two electrodes, the amplitude, duration and form of the deflections all enter; in other words the area bounded by the curves is the thing of real importance. Unfortunately, experiments involving dilatation of regional cardiac musculature and uncontrollable fluctuations of electrode pressure do not permit us to trust alterations in form or amplitude too implicitly, even though such changes be outstanding and though the experiments be performed with meticulous care. Consequently comparative measurements of surface areas may sometimes be doubtful. Regardless of form and amplitude variations, the duration of a state of potential difference can always be precisely determined by the time between the two reference points, *A* and *F* (segment I, fig. 1). We shall refer to this as the A–F interval and may state at this point, what will be enlarged upon later, that this interval affords a good index of alterations in the total area of the curve, even when curves are not recorded through thermionic valve arrangements. We consider this A-F interval of much greater significance than any determination of the entire monophasic cycle because of the many variations that occur as the descending limb approaches the isopotential base line and because of the many difficulties faced in selecting a termination point at this base line.

The effects of coronary occlusion. Typical records with directly inscribed data as to calculated A-F intervals are shown in figure 1. Segment I illustrates a control monophasic curve. Almost immediately after clamping the ramus descendens anterior the A-F interval is slightly reduced, and within a minute (segment II) the contour of the curve changes. More significant, however, is the reduction of the A-F interval by 0.055 second. Although the amplitude showed no reduction for about four minutes, and a more significant decrease six minutes after clamping (segment III), it is quite obvious that the area under the curve has decreased within a minute after interruption of circulation. At the six

minute mark, ventricular alternans developed, both beats showing a considerable diminution in amplitude. It is impossible to find any significant difference between the amplitudes of the large and small beat of the alternans couple, whereas the disparity in their A-F intervals and in the areas under the two curves is materially great. This brings out the close relationship between the A-F interval and the area. It is important to stress that the abbreviation of the A-F interval consistently present in all of our records is solely due to an earlier release of potential at *F*; in no instance was the relation of the rise at *A* altered with respect to the R deflection of the E.C.G. Immediately after release of the coronary clamp, the amplitude of the monophasic curves enlarged, and within two minutes a normal control amplitude was obtained. Segment IV three

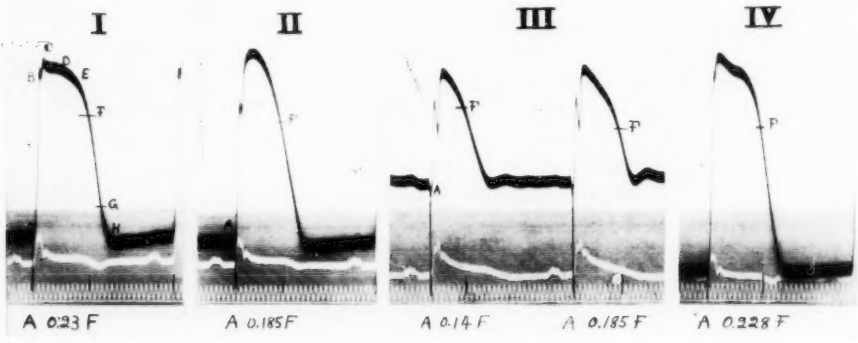


Fig. 1. Four segments of monophasic action potentials (upper larger curve) and E.C.G. lead II (lower). I, control; II, one minute after coronary occlusion; III, two beats of an alternans couple, six minutes after occlusion; IV, two minutes after release of coronary clamp. In all beats, heart cycles approximately equal (0.57 < 0.578). Time, 0.02 second.

minutes after restoration of circulation shows a curve with an amplitude, duration and form resembling that of the control, and the A-F interval lengthens to 0.228 second.

A brief enumeration of simultaneous electrocardiographic changes following coronary occlusion is of incidental interest. Segment II indicates that the immediate changes of the monophasic curve are just barely detectable in the E.C.G., the most conspicuous effect in this instance being a slight reduction in the negative T wave, which characterized the control record of this dog. Considerable elevation of the S-T segment occurred in an intervening record in which reduction of the A-F interval and of the potential area were the essential changes of the monophasic curve. Segment III presents the "high take off" and segment IV the return to control form after restoration of blood to the ischemic area.

These and other observations indicate that variations in the S-T segment begin with the decrease in duration of monophasic potential differences, and are only intensified as the amplitude actually decreases.

Of incidental interest also is the demonstration, only partially indicated by the segments selected for publication, that the sharp inflections at *A* and *B* appear progressively earlier in relation to the standard E.C.G. deflection and to the monophasic curves and that they are remarkably intensified as well. After the removal of the coronary clamp, they tend to diminish almost to the vanishing point.

Discussion. Although these experiments were completed a year ago, it seemed desirable to defer attempts at their evaluation or interpretation until additional knowledge was at our disposal. Meanwhile, Tennant and Wiggers (3) demonstrated that ventricular muscle fails to contract within one minute after coronary occlusion. Wilson et al. (4) pointed out that direct unipolar leads from the apex normally characterized by a transient upright spike, denoting rapid change of negativity beneath the exploring electrode, are transformed to a "broad downwardly directed monophasic or essentially monophasic deflection" within about a minute after ligation of the supplying artery. We should therefore expect our curves to be affected by such changes. The only change noted at this time is the typical abbreviation of the A-F interval and the reduction in area of our monophasic curve. This alteration is obviously governed by potential changes under the exploring electrode, for the right ventricular musculature is not involved, and it is exceedingly improbable that the dead tuberosity at the base of the right ventricle would show immediate changes in potential. Furthermore, in the two beats of an alternans couple (segment III) the two intervals vary definitely, although their amplitudes are identical.

This does not necessarily imply that monophasic curves are solely determined by the electrical variations under the exploring electrode on the ischemic area. One might equally well employ the conception of Wilson et al. (1) in explaining them—namely, that the same potential variations continue to occur during systole in the dead area (tuberosity on the right ventricle)—but that they are neutralized sooner as a result of a more persistent opposite potential in the ischemic area. The fact that the reduction of the A-F interval does not involve a delayed rise of the potential curve, but solely an earlier return toward isopotential, seems to favor such an hypothesis. On the other hand, it would be extremely awkward to interpret the immediate reduction of A-F intervals without significant reduction in amplitude on the basis that these monophasic curves are solely dominated by the changing potentials under the exploring electrode.

The interpretation adopted is, however, of far reaching significance in

explaining the persistence of good monophasic curves for many minutes through electrodal contacts with tissues in which contraction is known not to occur. If potential variations at the injured region are of no significance, then such persistence of monophasic curves would indicate that excitation of an ischemic area occurs without contraction. In this event monophasic curves derived from contracting muscle must be initiated by passage of impulses, whereas their perpetuation is materially modified by the contractile process. Such an interpretation, however, would be quite hazardous. All of our observations incline us to concede that they may be best explained by the assumption that the initial steep rise is due to potential changes at the original dead area. It follows that measurements of variations in the durations of monophasic cycles recorded without any reference curves—an E. C. G. for instance—could not present crucial evidence for the view that potential variations under the active electrode solely dominate the curve. Because of this, the much slighter differences in duration of the monophasic curves obtained by Jochim, Katz and Mayne (5) as a result of exploring numerous points on the ventricular surface could be explained equally well on the hypothesis that the initial portion of the normal monophasic curve is dominated by changes at the injured area, but that its duration is governed by potential changes at the exploring electrode.

SUMMARY. Monophasic curves, recorded from a dead basal region of the right ventricle and the initially normal apex of the left ventricle were obtained before and at various intervals following occlusion of the ramus descendens anterior.

In the analysis of such curves, emphasis is placed on two reference points—viz., the moment of initial rise, *A*, and the point of abrupt fall toward isopotentiality on the descending limb, *F*. Since the interval (A-F) largely determines the area bounded by the curves, it gives more exact information of changes at the exploring electrodes than does the amplitude or contour of curves that can be recorded.

The earliest and most outstanding alteration of direct monophasic leads following coronary occlusion is the reduction in the A-F interval, with consequent decrease in the area bounded by the curve. This reduction occurs without any amplitude diminution about a minute after occlusion and is due to an earlier termination of the potential difference between electrodes. These facts indicate that monophasic potential deviations are not free from potential changes at the initial dead area, but they strongly suggest that the duration and area of the curves are significantly affected by changes under the exploring electrode.

The general deduction is made that variations in the A-F interval or duration of the curve cannot be used as evidence that monophasic curves are due solely to potential changes under an exploring electrode, unless

it is shown that the rise and not only the termination changes with reference to deflections of a standard E. C. G.

A second change occurs in monophasic leads from 4 to 5 minutes after occlusion, i.e., 3 to 4 minutes after contractions have been arrested. It consists in a reduction in amplitude, without further abbreviation of the A-F interval and without change in the relation of the rise to Q-R-S complex of an E. C. G. This obviously denotes not only a greater but an earlier development of an oppositely directed potential under the exploring electrode in the ischemic area.

The fact that monophasic curves still appreciable in size can be derived from this area until the ventricles fibrillate is a second bit of evidence that the electrode on the original area of injury exerts an important influence on the curves.

In experiments in which coronary blood flow is restored previous to fibrillation, the monophasic curves revert to their original form. This indicates that the changes described are not those due to dead tissues but to tissue the function of which has been physiologically suspended.

CONCLUSION

Our results favor the interpretation of Wilson et al. that monophasic leads derived from an injured and an uninjured region of the ventricle are determined, to a significant extent, by alterations under the electrode at the injured surface, but that they also show that the duration of such curves and the areas bounded by them give significant information with regard to potential changes that occur in the region of the exploring electrode.

REFERENCES

(1) Wilson, Macleod and Barker. Proc. Soc. Exper. Biol. and Med. **27**: 588, 1930; **30**: 797, 1933; J. Gen. Physiol. **16**: 423, 1933; Univ. of Mich. Studies **10**: 1933, Univ. of Mich. Press, Ann Arbor.

(2) Schütz. Ztschr. f. Biol. **92**: 441, 453, 1932.

(3) Tennant and Wiggers. This Journal **112**: 351, 1935.

(4) Wilson et al. Trans. Assn. Am. Phys. **47**: 154, 1933.

(5) Jochim, Katz and Mayne. This Journal **111**: 177, 1935.

PHYSIOLOGICAL REVIEWS

Tentative Contents of Volume 16, 1936